

A Novel In Vivo Transcription Assay Demonstrates the Presence of Globin-Inducing *trans*-Acting Factors in Uninduced Murine Erythroleukemia Cells

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We report the development of a novel in vivo transcription assay for *trans*-acting factors regulating the human γ - and β -globin genes. A cDNA coding for the human tissue-type plasminogen activator (t-PA) was inserted into the globin genes. Simian virus 40 small T-antigen splice and polyadenylation signals were included to produce a mature transcript coding for t-PA, whose activity can be detected in single cells by a fibrin-agarose plaque assay. Stable murine L-cell transfectants of the γ .t-PA and β .t-PA hybrid genes were fused to various cell lines to show that t-PA expression is increased specifically by erythroid MEL, HEL, and K562 cell fusion. The analogous *H-2K^b*.t-PA construct was not inducible under the same conditions. Interestingly, uninduced MEL cells increased β .t-PA expression to the same extent as induced MEL cells. Chemiosmotic permeabilization of the β -globin tester cell line and exposure to nuclear extracts were used to assay for *trans*-acting factors capable of stimulating β .t-PA expression. Such factors were shown to be present in the nuclei of uninduced MEL cells.

The five active human β -globin-like genes form a cluster extending over 60 kilobases (kb) of DNA in the order 5'- ϵ - γ^G - γ^A - δ - β -3'. The genes are expressed in a developmentally and tissue-specific manner: the embryonic (ϵ) gene is expressed in primitive nucleated erythrocytes of the embryonic yolk sac, fetal (γ^G and γ^A) genes are transcribed in fetal liver, and adult (δ and β) erythropoiesis occurs in bone marrow (for a review, see reference 9). The control of globin gene transcription is believed to be achieved in part by the binding of *trans*-acting factors to *cis*-acting regulatory DNA sequences within the transcription unit. Globin gene transfer experiments in erythroid cell lines (6, 33) and microinjection in fertilized mouse eggs (21a, 22; R. R. Behringer, R. E. Hammer, R. L. Brinster, R. D. Palmiter, and T. M. Townes, Proc. Natl. Acad. Sci. USA) have identified *cis*-acting control sequences in both the 5' and 3' regions of the human β -globin gene. The introduced genes are regulated in the same manner as their endogenous counterparts, although they are physically unlinked. This indicates the existence of diffusible *trans*-acting factors, a conclusion that is supported by cell fusion experiments in which normally silent globin genes are activated in MEL-K562 heterokaryons prior to nuclear fusion (1).

To date, various in vitro approaches have been used to detect and facilitate the purification of *trans*-acting transcription factors, leading to the isolation of several in homogeneous form (4, 17). Such work has taken advantage of the sequence-specific DNA binding of factors to produce (i) altered electrophoretic mobility of DNA fragments (gel retardation) (13, 15), (ii) protection of bound sequences from chemical or enzymatic cleavage (footprinting) (14, 30), and (iii) factor immobilization on an affinity chromatographic column (19). In addition, stimulation of runoff transcription in extracts has proven a useful tool in factor purification (10). However, with the exception of an in vivo form of DNA

footprinting (8), none of these approaches can unequivocally elucidate the events of eucaryotic gene regulation that occur in the living cell.

We have developed an in vivo transcription assay for *trans*-acting factors with the following criteria. (i) The assay should be sensitive and, preferably, able to detect factors in single cells. (ii) The assay should not depend on a prior detailed characterization of *cis*-acting regulatory sequences. (iii) The assay should be reliable, fast, and easy to carry out. (iv) The assay should preferably be applicable not only for *trans* factor proteins, but also for their mRNA (and cDNA) for cloning purposes.

To achieve these goals, we have chosen the human tissue-type plasminogen activator (t-PA) gene as a marker gene. The t-PA cDNA has previously been used to assess transfection efficiency and promoter strength (20). Moreover, it can be detected in single cells or living colonies by a rapid assay with fibrin-agarose overlay and plaque formation. Most importantly, living cells can be recovered from the plaques for further characterization (18). We inserted the human full-length t-PA cDNA (5) into the human γ - and β -globin genes and the mouse *H-2K^b* major histocompatibility complex gene, to obtain t-PA transcription under the control of γ - and β -globin and *H-2K^b* *cis*-acting regulatory sequences. The hybrid genes were introduced into mouse L-cells to establish in vivo transcription tester cell lines. We show that nuclear factors can be introduced into these cells and increase t-PA production either by cell-cell fusion or directly by chemiosmotic permeabilization (25) and exposure to nuclear extracts. The results show the presence of globin-specific *trans* factors in erythroid-specific cells (MEL, HEL, and K562). In MEL cells, the factors are present even before the globin genes are expressed.

MATERIALS AND METHODS

Construction of hybrid genes. The t-PA cDNA was derived from the TRBM6 cell line (5) and was a gift from Beecham

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Pharmaceuticals Research Division. The plasmid was modified by insertion of a *Hind*III linker at the *Bam*HI site, from which t-PA cDNA and simian virus 40 (SV40) splice and polyadenylation sequences were excised as a *Hind*III fragment. This fragment was ligated in the correct transcriptional orientation into a *Hind*III linker, placed at the *Nco*I site of the human β - and γ -globin genes and *Nru*I site of the murine *H-2K^b* gene (Fig. 1). Human globin gene sequences were contained in plasmid pRT1 (16), which also carries the herpes simplex virus (HSV) thymidine kinase gene (*tk*) selectable marker. *H-2K^b* was cloned in pTM, which carries the neomycin resistance gene (16).

Cell culture. Mouse Ltk⁻, human HeLa, Bowes melanoma, and monkey Vero cells were grown in monolayer culture in Eagle minimal essential medium (E-MEM) supplemented with 10% foetal calf serum (FCS). Murine erythroleukemia (MEL) cells (clone C88, APRT⁻) were cultured in E-MEM with 10% FCS and 50 μ g of diaminopurine per ml and induced with 2% dimethyl sulfoxide at a density of 5×10^5 to 10×10^5 /ml for 48 h. K562 cells were cultured in E-MEM with 10% FCS, and HEL cells were cultured in RPMI 1640 medium with 10% FCS.

Transfection of Ltk⁻ cells. Transfections were performed with circular β .t-PApRT and linear γ .t-PApRT and *H-2K^b*.t-PApTM. Ltk⁻ cells were plated at a density of 10^5 per 90-mm dish the day before transfection. Calcium phosphate coprecipitates were prepared as described (31) with 20 μ g of DNA and 1 ml of precipitate per dish. For transfection, the medium was removed, precipitate was added, and the dish was left at room temperature for 20 min. Cells were then washed with medium and shocked in 15% glycerol-phosphate-buffered saline. β - and γ .t-PA transfectants were selected in HAT medium (100 μ M hypoxanthine, 0.4 μ M aminopterin, and 16 μ M thymidine), and *H-2K^b*.t-PA colonies were established in normal medium supplemented with G418 (500 μ g/ml).

S1 nuclease protection analysis of β .t-PA clones. Total RNA

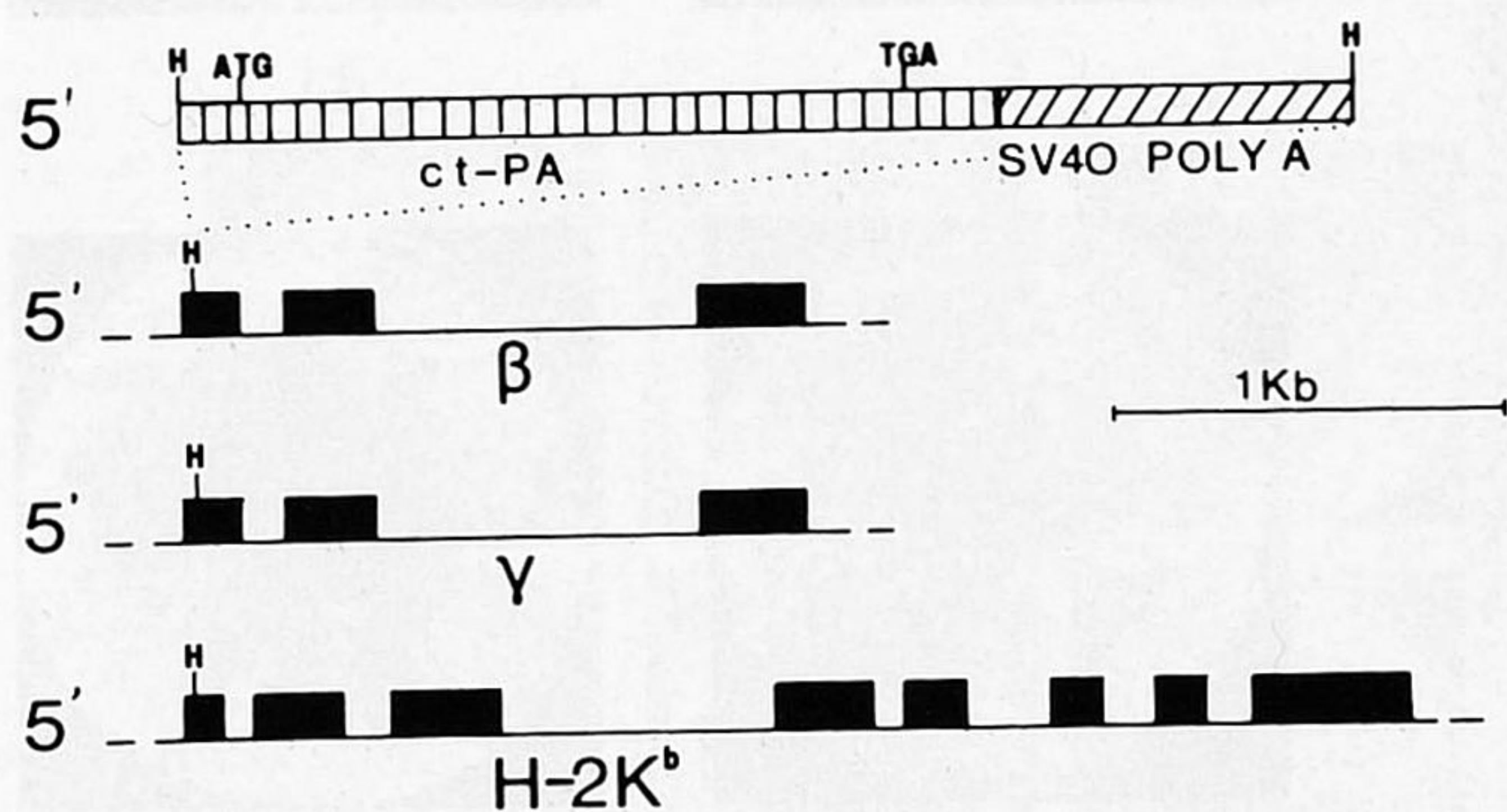


FIG. 1. Construction of the β -, γ -, and *H-2K^b*.t-PA hybrid genes. The t-PA cDNA (ct-PA) and SV40 splice and polyadenylation (polyA) sequences (Materials and Methods) were ligated as a 3.0-kb *Hind*III (H) fragment into the human β - and γ -globin and murine *H-2K^b* genes so that transcription and pre-mRNA processing would produce an mRNA coding for t-PA. A *Hind*III cloning site was provided in these genes by the insertion of a *Hind*III linker at the *Nco*I site of the β - and γ -gene (translation initiation site) and at the *Nru*I site of the *H-2K^b* gene (20 bp 5' to the translation initiation site). The globin gene fragments were a 4.7-kb *Bgl*II fragment and a 3.3-kb *Hind*III fragment carrying sufficient *cis*-acting regulatory sequences for regulated expression of the β - and γ -genes, respectively (32). For *H-2K^b*, a *Hind*II-*Eco*RI fragment was used (-101 deletion mutant) which has been shown to be uninducible in MEL cells, as is the *H-2K^b* gene with 2 kb of 5'-flanking sequences (33; M. Antoniou, unpublished data).

was isolated from β .t-PA clones as described (7), and 5' S1 nuclease protection analysis was performed (2) with the *Acc*I-*Bgl*II probe of the β .t-PA construct (see Fig. 2). A 30- μ g amount of RNA was analyzed per assay, and the DNA fragments protected from S1 digestion were analyzed by electrophoresis through a 6% urea-polyacrylamide gel and visualized by autoradiography at -70°C with intensifying screens.

Formation of heterokaryons by cell fusion. Cells were cultured carefully to be maximally viable before cell fusion. In particular, adherent cells were plated at 25% confluency the day before fusion to facilitate quick trypsinization and the recovery of single cells. Suspension cells were not trypsinized, as this did not appear to influence fusion efficiency, in agreement with a previous report (1). All cells were washed three times in E-MEM (room temperature) to remove all traces of serum, counted, and mixed in the appropriate quantities. We used a 100-fold excess of donor cells (10^7) to tester cells (10^5) per fusion to promote donor-tester fusion and minimize tester-tester fusion. Cells were pelleted, all medium was aspirated, and then the cells were gently suspended at 37°C in 100 μ l of a solution containing 50% polyethylene glycol (PEG) 1500 and 75 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5). After 30 s the reaction was halted by the addition of 10 ml of E-MEM at 37°C . Using this protocol, we have not encountered problems previously described for fusing cells in suspension (1). In our hands, approximately 10% of tester (L) cells could be fused to a wide variety of donor cells.

Detection of t-PA by fibrin-agarose overlays. Following fusion, a portion was removed for further culture. Cell viability was calculated from colony formation after 10 days (usually 50% of L-cells survived), and fusion efficiency was monitored by Hoescht 33258 nuclear staining as described (3) after 12 h in culture. The remaining cells were pelleted, the medium was removed, and the cells were suspended in 0.25 ml of 10- μ g/ml bovine fibrinogen (Sigma Chemical Co.) in phosphate-buffered saline (PBS, calcium- and magnesium-free) at 37°C . This was then quickly mixed with 2 ml of warmed basal E-MEM with antibiotics and 1.25% low-melting-point agarose supplemented with 25 mU of bovine thrombin (Sigma) and 20 mU of bovine plasminogen (Sigma) per ml. The cell suspensions were then pipetted onto a warmed 60-mm dish from which medium had just been aspirated. Agarose was allowed to set at room temperature, following which the dishes were incubated at 37°C in 5% CO_2 . Clotting occurred in approximately 10 min. Overlays of sticking colonies were performed after cells were washed three times in serum-free medium. In these cases, all the components of the fibrin-agarose overlay were mixed as one and then dispensed quickly.

Stimulation by nuclear extracts. Nuclear extracts were prepared from 10^9 uninduced MEL cells (11). A 0.3 M NaCl nuclear wash was precipitated with 0.35 g of $(\text{NH}_4)_2\text{SO}_4$ per ml and dissolved in 1 ml of 0.1 M NaCl-10% glycerol-RSB-1 mM dithiothreitol and dialyzed overnight against the same buffer. The final extract contained approximately 4 mg of total protein per ml. Uninduced T1 tester cells (β .t-PA) (10^7 cells) were harvested, washed (no serum), and suspended in 1 ml of 1.4 M glycerol-PBS and permeabilized as described with freshly prepared lysolecithin (*L*- α -lysophosphatidylcholine) (25). Samples (10 μ l, 10^5 L-cells) were added to 200 μ l of prewarmed (37°C) basal E-MEM plus antibiotics containing 0 to 50 μ g of nuclear extract. Cells were incubated for 10 min, suspended in fibrin agarose overlays as described above, and incubated at 37°C in 5% CO_2 for 80 h.

RESULTS

Construction and expression in mouse L-cells of a human β -globin-t-PA hybrid gene. The starting point for construction of the β -t-PA hybrid gene was β pRT (32), a plasmid containing the human β -globin gene as a 4.7-kb *Bgl*II fragment and an HSV *tk* selectable marker. This cloned β -gene is correctly regulated in transfected differentiating MEL cells, since it carries the 5' and 3' *cis*-acting elements required for erythroid-specific developmental regulation (33). Induction of MEL cells causes more than a 100- to 200-fold increase in β^{maj} transcription, accompanied by increased (>50-fold) transcription of the introduced human gene (32). The translation initiation codon of the β -gene was destroyed by insertion of a *Hind*III linker at the *Nco*I site, into which a t-PA cDNA was cloned (5). SV40 small T-antigen splice and polyadenylation sequences were included so that transcription of the hybrid gene and splicing would produce an mRNA containing the β -globin leader sequence, the t-PA coding region, and an SV40 3' region plus the poly(A) tail (Fig. 1). Similar constructs were made with the fetal human γ -globin gene and the nonerythroid-specific murine *H2-K^b* major histocompatibility gene. To character-

ize the system, the β -t-PA hybrid gene was introduced into mouse Ltk⁻ cells by calcium phosphate-glycerol shock (31), and stable transfectants were selected in HAT medium. Clones were picked, expanded, and analyzed for expression of the introduced DNA by 5' S1 nuclease protection analysis (2) and by fibrin-agarose overlays of colonies (18). The latter technique exploits the potency with which t-PA secreted from the living cell can initiate lytic plaque formation in an opaque fibrin-agarose matrix. Previous reports have demonstrated a linear relationship between t-PA production and area of fibrin plate lysis (18). Figure 2A shows an S1 nuclease protection assay of 10 independently transformed clones, 7 of which gave the expected 240-nucleotide fragment corresponding to correctly initiated β -t-PA mRNA. One of these seven (2.7) was found negative for t-PA production on colony fibrin-agarose overlay (not shown) and was discarded. Clone T1 gave the lowest level of protected fragment, and this corresponded to the lowest level of t-PA synthesis, only detectable above L-cell background on extended overlay incubation (Fig. 2B). It is important to note that these overlays were done on expanding colonies rather than single cells (see below). Clone T3 gave a higher S1 signal, corresponding to higher t-PA production in fibrin-

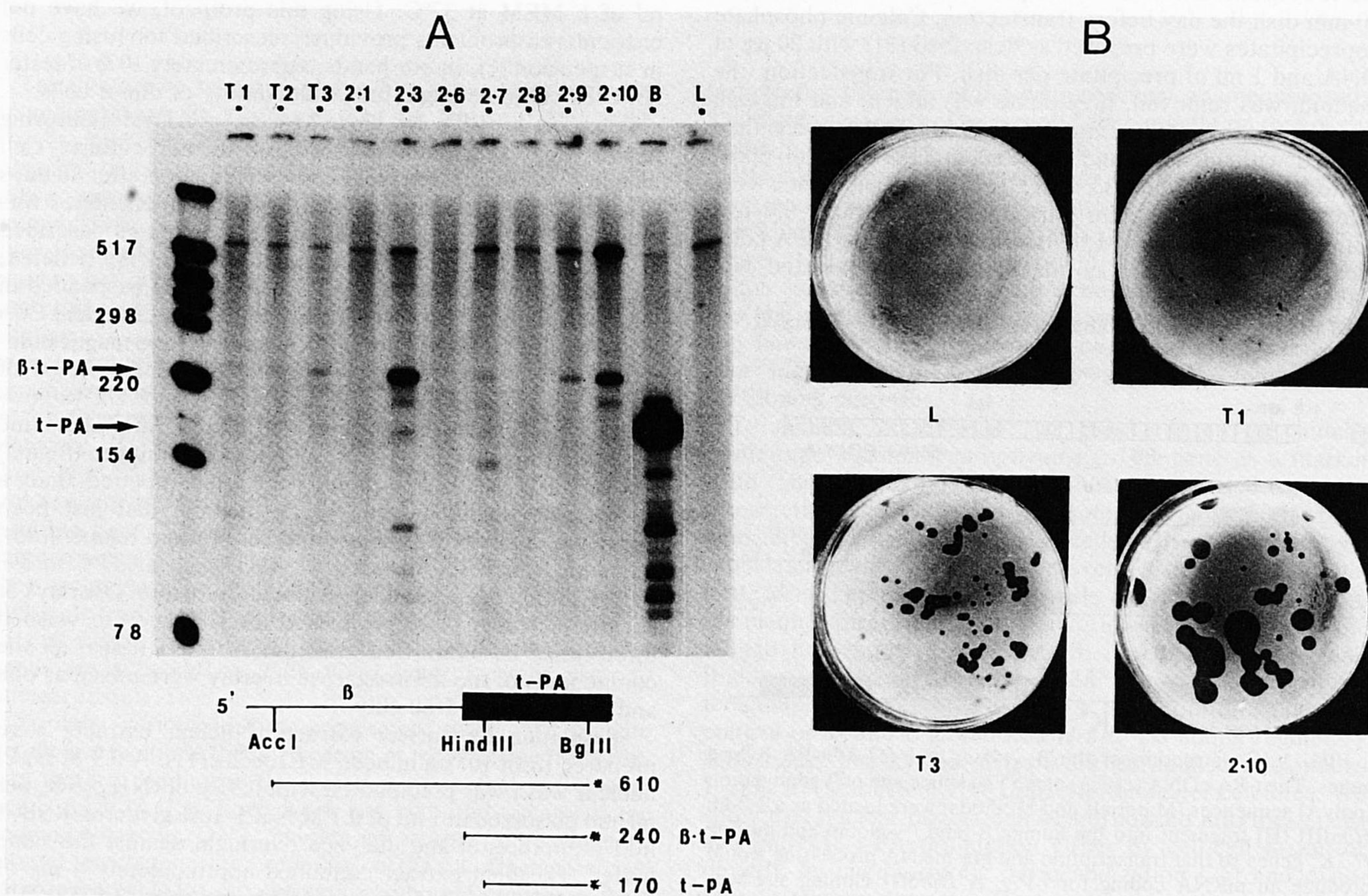


FIG. 2. Analysis of L-cell clones for β -t-PA expression. (A) A 5' S1 nuclease protection assay was performed on 30 μ g of total RNA from 10 L-cell clones carrying the β -t-PA construct (lanes T1 to 2.10). The 5'-end labeled probe used was as indicated below the gel, giving a 240-nucleotide fragment of β -t-PA (upper arrow) protected from S1 digestion by correctly initiated β -t-PA mRNA. Lane B is a control with 30 μ g of Bowes melanoma RNA (see text). The shorter protected fragment of t-PA (lower arrow) is due to the lack of β -globin leader sequences on the mRNA. Lane 12, 30 μ g of untransfected L-cell RNA. Marker sizes (pBR322 digested with *Hinf*) are indicated (in base pairs). (B) Fibrin-agarose overlays of 9-day-old L-cell colonies. Colonies were overlaid as described in Materials and Methods and incubated for 72 h at 37°C in 5% CO₂. Untransfected L-cells (L) showed no plaques, whereas β -t-PA clones T1, T3, and 2.10 showed lysis zones corresponding to t-PA synthesis from the β -t-PA gene. Increasing lysis correlated with increasing S1-protected signal (see panel A). Clone 2.10 was slower growing, and thus this colony assay underestimates t-PA production.

agarose overlay. Clone 2.10 had a still higher signal and produced the most t-PA. We therefore conclude that increased t-PA production, as assayed by fibrinolysis, is a reflection of elevated transcription and t-PA production, in agreement with earlier results (18, 20). Cell line T1 was considered a good candidate for a tester cell line, since it has very low levels of β .t-PA mRNA. This results in a low background of t-PA production, and this cell line would therefore be the most sensitive with which to measure induced transcription levels (see below).

Elevation of β .t-PA transcription by fusion to MEL cells. β .t-PA clones T1 and 2.10 were fused by using PEG 1500 to MEL cells (10^5 and 10^7 , respectively) in order to deliver *trans*-acting factors regulating the human β -globin gene and thus activate the construct. MEL cells were induced for 48 h prior to fusion, since we assume that accumulated β -globin-specific *trans*-acting factors are maximal at this time, i.e., preceding maximum globin mRNA levels. Induction was checked by incubating a sample for a further 48 h and testing for hemoglobin production (not shown). L-cell (tester line) viability was monitored by plating a small portion of the fusion culture. The formation of heterokaryons was monitored by nuclear staining (Hoechst 33258 [3]) and allowed an estimate of the number of MEL and L-cell nuclei per heterokaryon. Due to the 100-fold excess of MEL cells, we were able to fuse one or two MEL cells to approximately 10% of the L-cells, of which 50% survived PEG treatment. Since we used 10^5 L-cells per fusion, this resulted in approximately 5×10^3 viable L-cell-MEL cell heterokaryons per dish, which correlates well with the number of single L-cell (small) plaques observed in the overlay (Fig. 3). The L-cell lines T1 and 2.10 were tested in this assay, and both showed substantially elevated t-PA synthesis by cell fusion to induced MEL cells, as judged by fibrinolysis. The amount of elevated t-PA synthesis cannot be measured by direct nuclease protection analysis because very small numbers of cells (5×10^3) are involved. Instead, it was measured by comparison with a high t-PA mRNA-producing cell line (Bowes

melanoma) (27) rather than the original T1 or 2.10 cell line. T1, in particular, had such a low level of background t-PA synthesis that it took several days of overlay incubation to visualize it at all. It is therefore inaccurate and impractical to compare fused and nonfused T1 cells in the overlay. The 5×10^3 fused T1 (or 2.10) cells produced the same quantity of t-PA by approximately the same number of Bowes melanoma cells in the same time (Fig. 3 and 4). By comparison of the mRNA levels (Fig. 2) with those in T1, 2.10, and Bowes melanoma cells, there was at least a 100-fold increase of β .t-PA mRNA in fused cells over that in nonfused T1 cells. Such a large increase is consistent with the large increase of β -globin mRNA synthesis in MEL cells after induction of differentiation. The time course of plaque formation (plaques were evident after 12 h) indicated that activation occurred prior to nuclear fusion and, consequently, that *trans*-acting factors or mRNA was involved. Clearly these factors could be assayed conveniently and rapidly in single cells by using the t-PA cDNA as a marker gene. T1, containing one full copy of the β .t-PA construct (not shown), was chosen for further characterization; 2.10 was discounted due to its higher background of t-PA synthesis (Fig. 2).

Elevated transcription of the β .t-PA and γ .t-PA genes is stage and tissue specific. To determine whether the β .t-PA activation was erythroid cell specific and stage specific (fetal γ - versus adult β -globin), we used β .t-PA, γ .t-PA, and $H-2K^b$.t-PA tester cells in combination with a number of fusion partners; i.e., uninduced MEL cells, which transcribe transfected γ -globin, β -globin, and $H-2K$ genes (33); induced (differentiated) MEL cells, which transcribe significantly increased levels of the β -globin gene (33); and HEL and K562 cells (24, 28) which express embryonic ϵ - and fetal γ -globin genes. We have shown that K562 cells transcribe transfected γ -globin and $H2-K$ but not β -globin genes (21). In addition, we used a number of nonerythroid cell lines (e.g., HeLa). Eight $H-2K^b$.t-PA tester cell lines were used, and all gave the same result. Fusion with uninduced or induced MEL cells or any of the other cell lines showed no response in the fibrin-agarose single-cell overlays (Fig. 4), although these clones were shown to express the $H-2K^b$.t-PA construct by the overlay of expanding colonies (not shown). We therefore conclude that the assay is specific for erythroid cells and that posttranscriptional events (e.g., RNA stability) or the t-PA sequences themselves are not responsible for activation of the globin.t-PA gene upon cell fusion (Fig. 3 and 4).

When clone T1 (β .t-PA) was fused to HeLa cells and a monkey kidney cell line (Vero) at a fusion efficiency equivalent to that of induced MEL cells (Fig. 3), no increased t-PA synthesis could be detected by the fibrin-agarose plaque assay (Fig. 4, Vero cells not shown). Furthermore, human erythroleukemia (HEL and K562) cells did not activate the β .t-PA construct on fusion. In addition, we screened a variety of nonerythroid cell lines for T1 stimulation, but encountered high endogenous t-PA background problems from the fusion partner. Nevertheless, none showed an increase of t-PA production after fusion (not shown). Surprisingly, fusion to uninduced MEL cells gave activation very similar to that observed with induced MEL cells, and we conclude that the β .t-PA construct in clone T1 responds in a true adult-stage erythroid-cell-specific manner (MEL versus K562), although it does not mimic the increased expression of the β -globin gene observed after MEL cell differentiation (see Discussion).

In contrast to the T1 (β .t-PA) fusions, γ .t-PA tester cell lines, which all had a γ .t-PA background level before fusion,

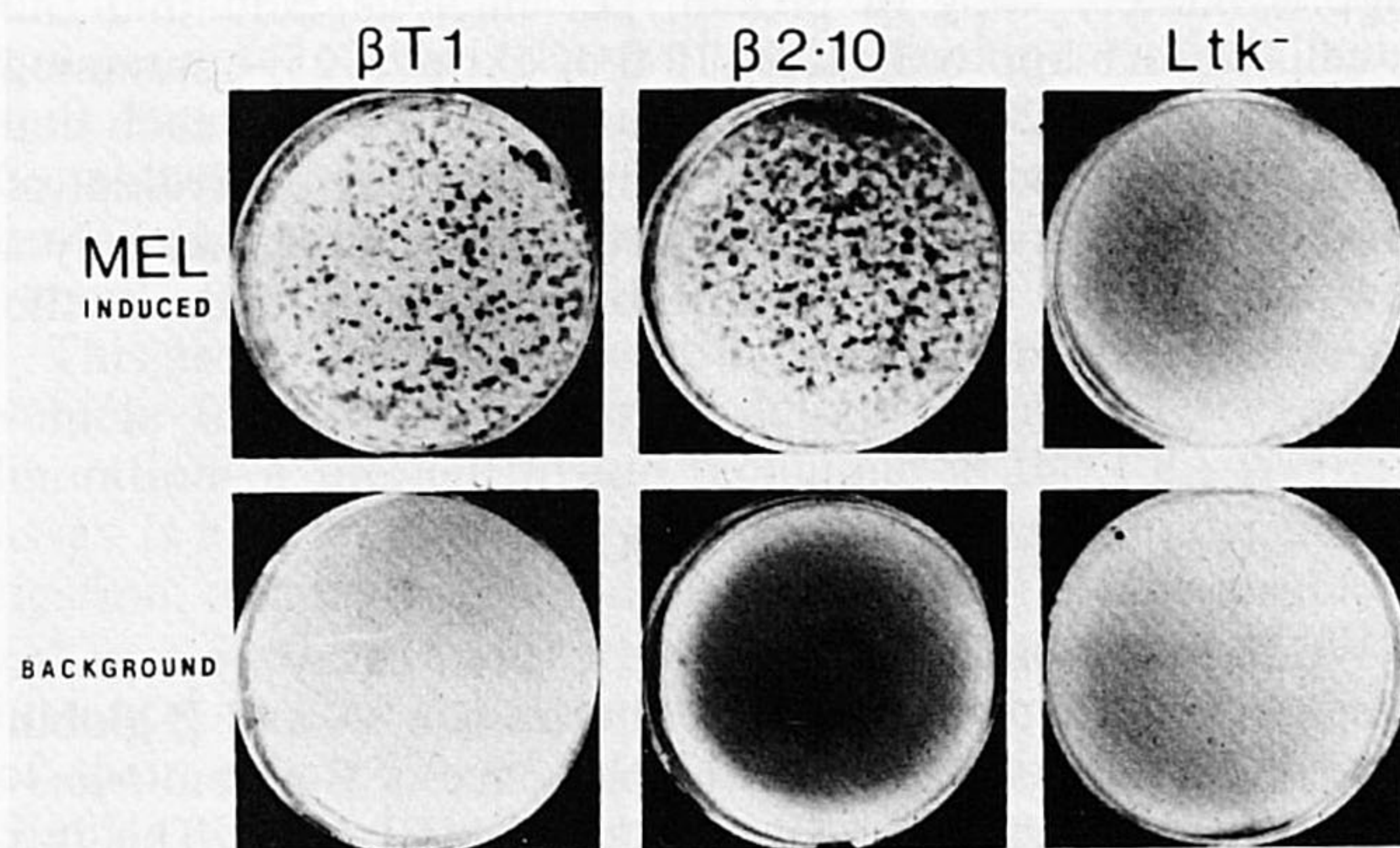


FIG. 3. Fusion with MEL cells causes increased expression of β .t-PA clones. Induced MEL C88 cells (10^7) were fused to 10^5 β .t-PA clone T1 and 2.10 cells and 10^5 untransfected L-cells as described in Materials and Methods. The bottom row shows the background from self-fusion of β .t-PA and L-cells alone (10^5 cells). Fusion products were assayed for t-PA production by fibrin-agarose overlay and incubated at 37°C for 18 h. Plates were then photographed with dark-field illumination. Both clones T1 and 2.10 showed elevated t-PA production, as assayed by plaque formation, when fused to induced MEL C88 cells. Untransfected L-cells did not respond to this assay, nor did self-fused β .t-PA clones (10^5 cells). Clone 2.10 gave some background in this single-cell fusion assay, as could be expected from the colony assay shown in Fig. 2.

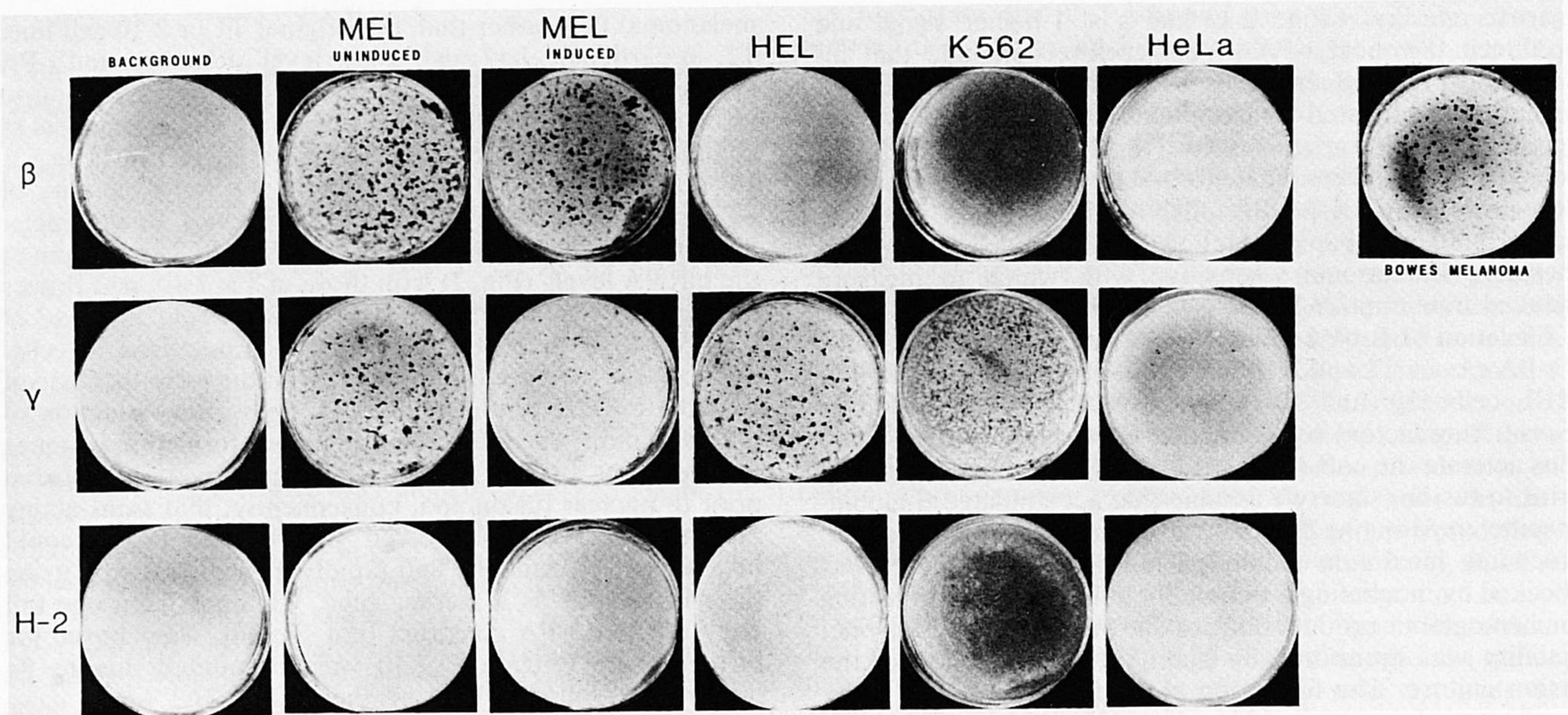


FIG. 4. Increased t-PA expression is gene and cell type specific, but does not mimic elevated β -gene transcription in induced MEL cells. L-cell clones carrying the β .t-PA construct (β), γ .t-PA construct (γ), and $H-2K^b$.t-PA construct (H-2) were fused to induced and uninduced MEL C88 cells, HEL cells, K562 cells, and HeLa cells and overlaid with fibrin-agarose. The first column shows the background from self-fusion of 10^5 β -, γ -, and $H-2K^b$.t-PA cells. Plates were incubated at 37°C in 5% CO_2 for 18 h and photographed with dark-field illumination. The β construct showed the same elevated expression on fusion to both induced and uninduced adult erythroid (MEL) cells, but not with fetal or embryonal erythroid (HEL and K562) or nonerythroid (HeLa) cells. Expression of the γ construct was increased by fusion to fetal embryonal and adult erythroid cells (HEL, K562, and MEL) (see text). The $H-2K^b$.t-PA construct did not respond. Plaque formation by 5×10^3 Bowes melanoma (control) cells under the same conditions is shown on the right.

showed a response to HEL and K562 as well as MEL cell fusion, in agreement with the results obtained in transfection experiments. Fusion with nonerythroid cell lines showed no effect. It is therefore possible to distinguish between fetal γ - and adult β -globin-specific transcription, and we conclude that the globin.t-PA transcription assay is erythroid and cell stage specific. Similarly, although not tested, we presume that the $H2-K^b$.t-PA cell lines can be used to study $H2-K$ gene transcription.

trans-Acting factors present in an uninduced MEL nuclear extract can increase β .t-PA expression. Using a recently developed protocol (25), we introduced uninduced MEL nuclear proteins into the β -tester line T1 to assay for *trans*-acting factors regulating the human β -globin genes. β .t-PA tester cells (T1) were permeabilized by incubation in glycerol-PBS-lysolecithin. Samples (10^5 cells) were incubated in serum-free medium in the presence of increasing amounts of MEL nuclear extract or fluorescein isothiocyanate-labeled bovine serum albumin as detailed in Materials and Methods. Cells were then either suspended in fibrin-agarose and incubated to measure t-PA activity or examined by fluorescence microscopy to monitor protein permeability (25). Conditions were optimized for L-cell permeabilization and recovery of viable cells (25) (Materials and Methods), giving 100% permeabilization and 10% cell survival (i.e., 10^4 of 10^5 treated cells). Figure 5 shows the result of fibrin-agarose assays of permeabilized β -tester cells (T1) exposed to uninduced MEL nuclear extracts. Increasing plaque number corresponded to increasing amounts of extract. Permeabilized, nontransfected L-cells showed no response under identical conditions, nor did nonpermeabilized T1 tester cells (10^4) exposed to 50 μ g of extract. More importantly, a HeLa cell nuclear extract did not show any response either. Expression of the β .t-PA construct was therefore elevated in

response to (specific) *trans*-acting factors present in the uninduced MEL nuclear extract. Boiling the extract extinguished any response. At present it is very difficult to quantitate this experiment. We do not know the minimum nuclear concentration of factor required for activity, nor do we know the loss of activity in the extracts. Nevertheless, assuming that we have no loss of activity in the extract (approximately 4 pg of protein per nucleus) and that the L-cells absorb approximately 10 fl of extract (25)-containing medium (50 μ g/200 μ l), it can be calculated that each cell takes up about only 0.1% of a normal nuclear equivalent of protein. It is therefore perhaps not unreasonable that only a few hundred of the 10^4 permeabilized viable cells in the overlay produced a plaque.

DISCUSSION

We have developed a sensitive *in vivo* assay system for transcription factors regulating the human γ - and β -globin gene and have demonstrated the presence of β -globin *trans*-factors in uninduced as well as induced MEL cells. This is a surprising result and raises important questions concerning the mechanism of β -globin gene regulation (see below).

The *in vivo* assay system has a number of features that should make it widely applicable to many other gene families. First, it is not necessary to know the precise locations of the *cis*-acting regulatory sequences in the gene of interest. We used the entire β -globin gene as a fragment containing *cis*-acting sequences sufficient for regulated expression in MEL cells (33). This is in contrast to other methods for factor detection that use short DNA probes containing defined *cis*-acting elements (13). In these cases much initial work is needed to pinpoint important regulatory sequences.

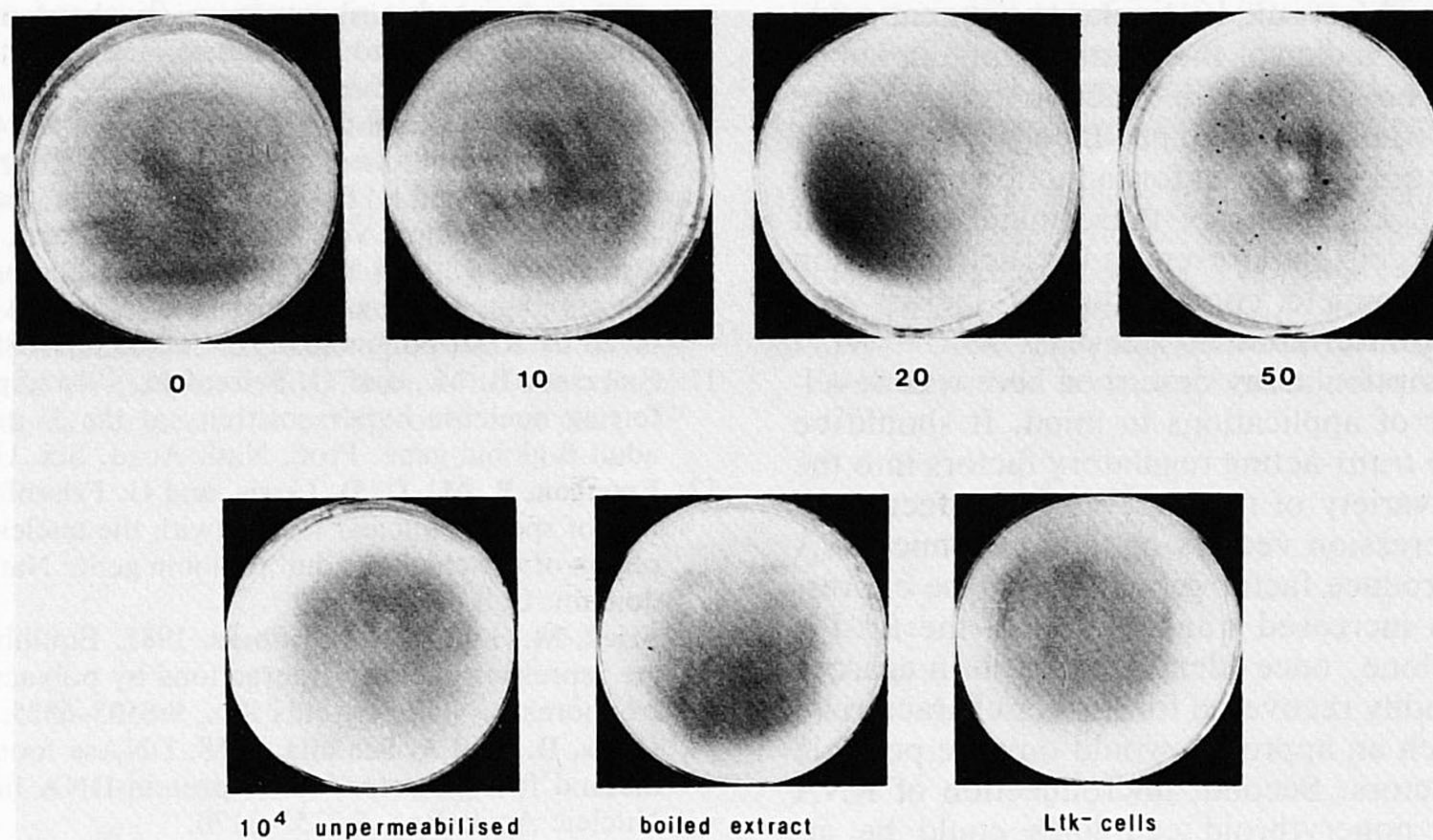


FIG. 5. β .t-PA activation by uninduced MEL nuclear extracts. The β .t-PA-containing tester L-cell line T1 was reversibly permeabilized (25), incubated with increasing amounts of nuclear extract (0, 10, 20, and 50 μ g) from uninduced MEL cells, and suspended in fibrin-agarose. Plates were incubated at 37°C in 5% CO₂ for 80 h. Increasing plaque formation corresponded to exposure to greater quantities of nuclear extract. Totals of 0, 0, 70, and 200 plaques were visible with 0, 10, 20, and 50 μ g of extract, respectively (the smaller and fainter plaques were lost in the photographic reproduction). We used as controls nonpermeabilized T1 cells (10⁴ cells with 50 μ g of extract), 10⁵ permeabilized T1 cells with 50 μ g of boiled extract, and 10⁵ permeabilized Ltk⁻ cells (not containing the β .t-PA construct) with 50 μ g of extract. All of these had zero plaques.

Second, in our system transcription occurs inside the living cell, where the template is native chromatin. The level of transcription is then modulated *in vivo* by the addition of nuclear factors. This differs markedly from *in vitro* transcription in nuclear extracts. Third, the assay is sensitive, convenient, and rapid. Last, results obtained from *in vitro* DNA-binding studies must be qualified if their biological significance is to be accepted. For example, if a DNA-binding *trans*-acting regulatory factor possesses at least two domains, one of which is responsible for binding to DNA in a sequence-specific manner while the other interacts with components of the transcriptional machinery, then band shift experiments and footprinting do not provide a functional assay. In contrast to this, the use of an *in vivo* functional assay yields data of immediate biological relevance.

This assay was developed by using cell fusion as an initial vehicle for introducing *trans*-acting factors. However, a limitation of the cell fusion technique in the t-PA overlay assay is background t-PA synthesis in the cells under investigation, although it should be pointed out that this problem can be solved by the use of nuclear extracts (see below). For instance, we found that a number of cells produced sufficient of their own t-PA to interfere with a conclusive result. Similar to previous reports of the activation of muscle-specific genes in nonmuscle nuclei introduced into stable multinucleated myotubes (3), we have demonstrated the presence of factors regulating the human β -globin gene in both induced and uninduced MEL cells (see below). Since transcription of the β .t-PA construct was elevated prior to nuclear fusion, these factors must operate *in trans*. This was conclusively shown by the nuclear extract transfer experiments. We have also shown that increased expression of the β .t-PA construct was cell type specific; of the various lines tested, only MEL cells, in which the adult murine β -globin genes are expressed, contained factors capable of increasing expression of the human β -globin gene. This finding is

similar to recent data describing the activation of globin gene expression in transient erythroid-erythroid and erythroid-nonerythroid heterokaryons (1). However, the β .t-PA gene in our tester cell line was transcribed at a low level before cell fusion and thus reported solely on factors able to increase expression of an already activated gene. This can be measured by increased plaque formation relative to a known standard (e.g., Bowes melanoma cells). From this comparison, RNA levels in individual cells can be calculated.

We were surprised to find equivalent stimulation of the β -tester line by fusion to both induced and uninduced MEL cells, which strongly suggests that the specific *trans*-acting factors are present in MEL cells before induction of transcription of the endogenous adult murine β -genes. This is supported by the finding that the uninduced MEL nuclear extract activated the tester line (Fig. 5). Various mechanisms can be put forward to account for this. First, although unlikely, a low level of active factor (present in all MEL cells before induction) might be sufficient to stimulate the tester cell line maximally (at least 100-fold). If this were the case, an increased amount of factor (after MEL induction) would then be incapable of increasing t-PA protein product beyond this maximum (Fig. 3). (Simple calculations excluded the possibility that tester line activation by fusion to uninduced MEL cells was due to the presence of a small subpopulation [e.g., 1%] of fully induced MEL cells in the induced population, since this would result in fewer plaques than were observed.) Second, some of the factor(s) might be present before MEL cell induction in an active form, but the murine adult β -genes in uninduced MEL cells are inaccessible to the factor(s). The β .t-PA construct in the tester cell line, however, was already transcriptionally active due to transfection and selection procedures and could therefore respond to the factors. Third, and equally likely, the factor(s) might be present before induction but in an inactive or sequestered form. In the tester line, these factors might be modified by an activity constitutive to the L cell, resulting in elevated

β .t-PA transcription. This would be similar to a recent study in which a *trans*-acting factor of the immunoglobulin kappa gene was found to be present (in inactive form) before stimulation of kappa transcription in pre-B cells (29). Last, if a combination of factors is required for induction of β -globin transcription in MEL cells, one of these might be absent from uninduced MEL cells but present in L-cells. The fusion would result in a complete combination of factors and induction of β .t-PA transcription.

The *in vivo* transcription assay described here was developed with a number of applications in mind. It should be possible to introduce *trans*-acting regulatory factors into the tester cell line by a variety of routes. First, transfection of cloned cDNA in expression vectors or total genomic DNA could be used to introduce factor genes (23) whose expression would result in increased transcription of the β .t-PA construct. Such a clone, once identified by fibrin-agarose overlay, could be readily recovered for further characterization (18). Clearly such an approach would only be possible for single protein factors. Second, microinjection of RNA from erythroid and nonerythroid cell lines could be attempted. The assay for t-PA is extremely sensitive, and we have demonstrated that the tester line can be greatly stimulated to produce t-PA by cell fusion or nuclear extracts. Thus, few injected cells should be required under optimum RNA, microinjection, and overlay conditions. If successful, mRNA could be highly purified and cloned (into expression vectors). Third, the tester line clearly responded to nuclear extracts from the correct erythroid stage, suggesting that the *trans*-acting factors are proteins. These results have shown that this is a feasible approach and that *trans*-acting factors regulating the human β -globin gene could be purified from a low-salt-wash extract of the uninduced MEL nuclei. This finding is consistent with reports in which chicken β -globin gene factors have been identified in similar extracts from immature chicken erythrocytes (12, 26).

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