Temperature-sensitive *v-sea* transformed erythroblasts: a model system to study gene expression during erythroid differentiation

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The isolation and characterization of a temperature-sensitive mutant (*ts1* S13) of the avian erythroblastosis virus, S13, is described. The temperature-sensitive lesion in *ts1* S13 was identified as affecting the tyrosine kinase activity but not the plasma membrane localization of the *ts1* S13 *v-sea* gene product. Erythroblasts transformed by *ts1* S13 can be induced to synchronously differentiate into erythrocytes in an erythropoietin (EPO)-dependent fashion. Analysis of erythrocyte-specific gene expression in *ts1* S13 erythroblasts reveals that the transformed, self-renewing erythroblasts obtained at permissive temperature already express all erythrocyte genes tested for, although at a low level. Upon differentiation induction, expression of erythrocyte-specific genes is not coordinately regulated but rather involves complex regulatory mechanisms that appear to be specific for the individual genes.

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Erythroid differentiation is a particularly prominent and well-studied example of how the pattern of gene expression changes during terminal differentiation. During maturation of erythroid progenitors into erythrocytes, relatively few genes such as those encoding globins, cytoskeletal proteins, and erythrocyte-specific enzymes are induced to be expressed at very high level, whereas expression of a multitude of other genes (including those encoding 'housekeeping proteins') is shut down or diminished (for review, see Harrison 1984). Unlike other in vitro differentiation systems (e.g., myoblasts), in which the progenitors become postmitotic before reprogramming of the gene-expression pattern occurs, maturing avian erythroid progenitors (colony forming unit erythroid, CFU-E) undergo four to five cell divisions while actively accumulating red cell proteins (Holtzer et al. 1972). Therefore, analysis of red cell differentiation is a particularly well-suited system in which to study the regulation of tissue-specific gene expression (Choi and Engel 1986; Emerson et al. 1985). In addition, it promises to yield new insights into the interdependence of proliferation and differentiation (Falcone et al. 1984; Blau et al. 1985).

Erythroid progenitors cannot be purified in sufficient quantities for biochemical or molecular studies (van

Zant and Goldwasser 1984). Thus, gene expression during erythroid differentiation has mainly been studied in immature red cells from chick embryos (Blikstad et al. 1983), reticulocytes from anemic animals (Bonanov-Tzedaki et al. 1981), or erythroleukemic cell lines of mouse (Friend erythroleukemia cells) or human (K562, KD) origin. However, the first two approaches preclude analysis of very early, dividing progenitor cells such as CFU-Es, whereas the drug-induced differentiation of erythroleukemic cell lines is usually incomplete and independent of erythropoietin (Houseman et al. 1978; Marks and Rifkind 1978).

Some progress towards overcoming these problems was made by using clones of avian erythroid progenitors transformed by temperature-sensitive mutants of the avian erythroblastosis virus (AEV) (Graf et al. 1978). These AEV mutants encode a thermosensitive *v-erbB* protein exhibiting a single amino acid exchange in the *erbB*-kinase domain (Choi et al. 1986; Scotting et al. 1987), as well as an apparently unchanged *v-erbA* protein. When erythroblasts transformed by these temperature-sensitive (*ts*) AEV mutants are shifted to the non-permissive temperature, the proliferating, erythropoietin (EPO)-independent erythroblasts are both induced to differentiate into erythrocytes within 4–5 days and

become dependent on EPO for survival and differentiation (Beug et al. 1982; Beug and Hayman 1984). However, we have found recently that the *v-erbA* oncogene, a mutated version of the thyroid hormone receptor (Sap et al. 1986), drastically affects the transcription of certain erythrocyte-specific genes, leading to a differentiation arrest of temperature-induced temperature-sensitive AEV cells under certain conditions (M. Zenke et al., in prep.). In addition, the temperature-sensitive lesion in the *v-erbB* protein affects its cell-surface expression but does not abolish its tyrosine kinase activity, leading to a detectable 'leakiness' of the temperature-sensitive-*erbB* mutation (H. Beug et al., in prep.).

These problems were successfully overcome by use of a temperature-sensitive mutant of the avian erythroblastosis virus S13 (Benedict et al. 1985; Beug et al. 1985b). Here we describe the characterization of this mutant (*ts1* S13) and demonstrate its utilization in studying erythrocyte-specific gene expression during terminal differentiation. The *v-sea* oncogene product (gp155/70^{env-sea}), represents a tyrosine kinase domain that is fused to the carboxyl terminus of the viral envelope glycoprotein (Hayman et al. 1985). We will demonstrate that the thermosensitive lesion in the *ts1* S13 gp155/70^{env-sea} proteins causes an almost complete inactivation of their associated kinase activity, thus leading to a nonleaky temperature-sensitive phenotype of the mutant in both fibroblasts and erythroblasts. Analysis of erythrocyte protein and RNA expression during EPO-dependent, synchronous differentiation of these *ts1* S13 cells at 42°C reveals that self-renewal of the transformed cells at 37°C is compatible with low-level expression of most, if not all, erythrocyte-specific genes. Terminal differentiation, on the other hand, is characterized by a massive increase in transcription of some, but not all of the erythrocyte-specific genes studied.

Results

ts1 S13 exhibits a thermosensitive phenotype in fibroblasts

The *ts1* S13 mutant was detected as a spontaneous mutant upon biological cloning of several S13 isolates in nonproducer erythroblasts (Beug et al. 1985b). Upon repeated recloning, the temperature-sensitive phenotype of the *ts1* S13 mutant remained associated with this particular virus isolate, whereas erythroblasts transformed with several other S13 substrains exhibited a wild-type phenotype (Graf et al. 1978; P. Vogt and H. Beug, unpubl.). To study whether the *ts1* S13 virus would exhibit a temperature-sensitive phenotype in chick embryo fibroblasts (CEFs), *ts1* S13- and wt-S13-virus-transformed CEFs were assayed for the expression of several fibroblast transformation parameters (Royer-Pokora et al. 1978; see Materials and methods). Table 1 shows that *ts1* S13 fibroblasts maintained at 42°C for 24 hr were essentially indistinguishable from normal helper virus-infected fibroblasts, except for a slightly enhanced capacity to form minute colonies in soft agar. At the per-

missive temperature (37°C), *ts1* S13 fibroblasts exhibited a strongly transformed phenotype, characterized by fusiform morphology, absence of actin filament bundles, greatly reduced fibronectin network, strongly enhanced hexose transport rate, and efficient colony formation in soft agar. Except for some difference in hexose transport rate, the respective wt S13-transformed fibroblasts exhibited a strongly transformed phenotype at both 37°C and 42°C (Table 1). Thus, in contrast to the partial temperature-sensitive phenotypes observed with the available temperature-sensitive AEV mutants (Beug and Graf 1980; Palmieri et al. 1982), the *ts1* S13 virus induces a complete temperature-sensitive phenotype in CEFs.

Synchronous, hormone-dependent differentiation of ts1 S13 erythroblasts at the nonpermissive temperature

To isolate well-differentiating *ts1* S13 erythroblast clones exhibiting an in vitro life span long enough to allow production of erythroid cells at different stages of maturation in sufficient numbers for biochemical experiments, more than 100 *ts1* S13-transformed clones (Beug et al. 1985b) were screened for completeness of terminal differentiation, yield of differentiated cells, and in vitro life span, as described previously (Beug and Hayman 1984; Kowenz et al. 1987). In contrast to temperature-sensitive AEV clones, all temperature-sensitive S13 erythroblast clones tested exhibited essentially complete differentiation after 3–4 days at 42°C, with the viable cell population consisting of more than 95% mature cells (erythrocytes plus late reticulocytes). However, considerable variation was seen between different clones with respect to in vitro life span (15 to >40 population doublings in culture, clones with high life span occurring more infrequently), proportion of viable differentiated cells (20% to ≥95% after 3 days), and yield of differentiated cells (0.5×10^6 to 5×10^6 cells obtained from 1×10^6 cells induced to differentiate).

In Table 2 and Figure 1, the properties of two *ts1* S13 clones selected for a particularly long life span and high yield of differentiated cells are shown. Both clones exhibited an in vitro life span of more than 40 generations in culture. Mass populations shifted to the nonpermissive temperature in the presence of EPO contained more than 90% viable cells after 3 days at 42°C (Table 2) and yielded 3×10^6 to 5×10^6 mature cells per 10^6 induced cells (data not shown). Thus, these clones could be used to produce homogeneous populations of 10^7 to 10^8 erythroid cells at different stages of maturation when shifted to 42°C for various lengths of time, as demonstrated by morphology, hemoglobin content, and size distribution (Fig. 1).

ts1 S13-transformed erythroblasts were also clearly dependent on chicken EPO for survival, proliferation, and differentiation. Studies using more than 2000-fold purified chicken EPO, representing an acidic glycoprotein of approximately 30,000 daltons (Kowenz et al. 1987), clearly show that chicken EPO stimulates both proliferation and hemoglobin accumulation of temperature-induced *ts1* S13 cells (Fig. 2; Table 2). In contrast to

Table 1. Transformation parameters of ts1 S13- and wt S13-transformed fibroblasts

Virus	Temperature (°C)	Morphology	Actin cables (% positive)	Fibronectin	Hexose uptake (-fold stimulation) ^a	Agar colonies (cfu/ml)
ts1 S13	37	transformed	15	—	5.7	5.1×10^5
	42	normal	80	+++	1.2	3×10^3
wt S13	37	transformed	17	—	5.3	3.4×10^4
	42	transformed	17	—	3.1	1.5×10^4
RAV-1	37	normal	87	+++	1	$<10^2$

For definition of normal and transformed morphology, expression of actin filament bundles, and fibronectin network, see Royer-Pokara et al. (1978) and Palmieri et al. (1982). Hexose uptake data are the mean of three independent determinations. Agar colony formation was determined from counting >400 colonies from at least four separate dishes, using 10-fold serial dilutions of virus.

^a Relative to RAV-1-infected fibroblasts.

temperature-sensitive AEV cells (Beug et al. 1982), ts1 S13 cells that survived cultivation at 42°C in the absence of EPO still completely differentiated into erythrocytes (Table 2 and data not shown). This indicates that chicken EPO, like other hematopoietic growth factors, is required for survival and proliferation of differentiating precursors but does not appear to control the erythroid differentiation program per se.

The thermosensitive lesion in the gp155/gp70^{env-sea} transforming protein of ts1 S13 affects kinase activity but not intracellular localization

Biosynthesis of the *env-sea* fusion protein of S13 virus starts with a gp155^{env-sea} glycoprotein localized in intracellular membranes (Hayman et al. 1985). Similar to processing of the retroviral *env* precursor gp92 into gp85^{env} and gp37^{env}, the gp155^{env-sea} precursor is cleaved into the transmembrane protein gp70^{env-sea} and mature gp85^{env}, leading to insertion of gp70^{env-sea} into the plasma membrane (Hayman et al. 1985 and unpubl.). To study whether the temperature-sensitive lesion in ts1 S13 would affect processing and plasma membrane localization of the ts-gp155/gp70^{env-sea} protein or, alternatively, would render its associated tyrosine kinase activity

thermosensitive, pulse-chase analysis of [³⁵S]methionine-labeled ts1 S13 and wt S13 fibroblasts maintained at 37°C or shifted to 42°C was performed. Aliquots of the same cell preparations were also tested for in vitro kinase activity, using the immune complex autophosphorylation assay (Hayman et al. 1985, 1986). The results obtained show that gp155^{env-sea} was processed very inefficiently into gp70^{env-sea} at permissive as well as nonpermissive temperature in both ts1 S13 fibroblasts (Fig. 3A) and wt S13 fibroblasts (data not shown). However, autophosphorylation of both gp155^{env-sea} and gp70^{env-sea} was clearly thermolabile in ts1 S13 fibroblasts (Fig. 3A). As revealed by scanning of different exposures of the gel shown in Figure 3A, the kinase levels of gp155^{env-sea} and gp70^{env-sea} were reduced at least 5-fold and 20-fold respectively. In contrast, only a 2.5-fold decrease in autophosphorylation of gp70^{env-sea} was seen in wt S13 cells maintained at 42°C, whereas the kinase activity of the gp155^{env-sea} protein remained unchanged. The observed decrease in autophosphorylation of wtS13 gp70^{env-sea} was a reproducible effect, although other experiments resulted only in a 1.5- to 2-fold reduction in activity. This is in accord with the weak temperature sensitivity of hexose uptake in wt S13 fibroblasts and suggests that gp155^{env-sea} might be involved in fibroblast transformation.

The temperature-sensitivity of kinase activity of the ts1 S13-transforming proteins was even more striking in erythroblasts. In these cells, gp155^{env-sea} is processed rather rapidly into gp70^{env-sea}, and processing seems to be somewhat inhibited at 42°C in ts1 S13 erythroblasts but not in wt S13 erythroblasts (Fig. 3B; J. Knight and M. Hayman, unpubl.). Figure 3B demonstrates that the in vitro kinase activity of both gp155^{env-sea} and gp70^{env-sea} was drastically reduced (>10-fold and >30-fold, respectively) if the cells were kept at 42°C for 24 hours. Likewise, gp70^{env-sea} could be efficiently labeled in vivo with inorganic phosphate at 37°C, and this labeling was again reduced more than 10-fold after 24 hours at 42°C. Control experiments performed with wt S13-transformed erythroblasts revealed that autophosphorylation of gp155^{env-sea} and gp70^{env-sea} was only slightly reduced (<3-fold) by shift to the nonpermissive temperature (data not shown).

Table 2. Properties of ts1 S13- and wt S13-transformed erythroblast clones

Virus	Clone no.	Temperature (°C)	Percent LR + E		Percent viable cells	
			+EPO	-EPO	+EPO	-EPO
ts1 S13	A4	37	14	15	96	86
		42	>98	>98	88	44
	H2	37	3	5	80	76
		42	>98	>98	93	25
wt S13	A1	37	7	8	90	92
		42	10	12	85	81
	B3	37	1	1	94	96
		42	2	3	90	84

Percentages of late reticulocytes (LR) and erythrocytes (E) were determined in stained cytopsin preparations as described earlier (Beug et al. 1982). The percentage of viable cells was determined as in Schmidt et al. (1986c).

Figure 1. Synchronous, temperature-induced differentiation of *ts1* S13-transformed erythroblasts. *ts1* S13 erythroblasts (clone A4) were grown at 37°C or shifted to 42°C for the times indicated, purified (see Materials and methods), and analyzed for morphology and hemoglobin content by histochemical staining (left panels) or for their size distribution (right panels) using a Coulter counter (see Material and methods). Photographs (left panels) were taken under blue light (Beug et al. 1982) to reveal histochemical staining for hemoglobin. (Inset, bottom left panel) Terminally differentiated *ts1* S13 cells photographed under green light to reveal histological details.

Taken together, our results clearly demonstrate that the putative temperature-sensitive lesion in the gp155/gp70*env-sea* proteins of *ts1* S13 affects their autophosphorylation on tyrosine both in vitro and in vivo but does not grossly alter the processing and maturation of the *env-sea* fusion proteins. The latter conclusion was confirmed by studying cell-surface expression of the gp155/70 *env-sea* protein in *ts1* S13-transformed erythroblasts and fibroblasts, using a tumor-bearing rat serum to gp155/70*env-sea* that recognized an external *env*-determinant specific for the S13-transforming protein (Hayman et al. 1985). Figure 4 demonstrates that gp155/70*env-sea* behaves differently than gp70 *erbB* (Beug and Hayman 1984) in that these proteins continued to be ex-

Figure 2. Proliferation and hemoglobin production of differentiating *ts1* S13 cells are erythropoietin-dependent. *ts1* S13 cells were grown at 37°C (upper panel) or shifted to 42°C (lower panel) in the presence of different concentrations of >2000-fold purified chicken EPO and assayed in the DNA synthesis assay (●) or the photometric assay (▲) for EPO activity as described in Kowenz et al. (1987). As controls (○, △), inactive fractions from the final reverse-phase HPLC run (Kowenz et al. 1987) were included in the test.

Figure 3. Tyrosine kinase activity, but not processing of the gp155/70^{env-sea} protein is thermosensitive in *ts1* S13-transformed fibroblasts and erythroblasts. (A) *ts1* S13 (RAV-2) (*ts sea*) and *wt* S13 (RAV-2) (*wt sea*) fibroblasts were grown at 37°C or 42°C for 2 days and pulse-labeled with [^{35}S]methionine (^{35}S Met) for 20 min (P) followed by a chase period in cold medium of 150 min (C). Aliquots of the same cells were subjected to an in vitro immune complex tyrosine kinase assay (Hayman et al. 1985). The positions of the immunoprecipitated *env* precursor pr92^{env} and of gp155^{env-sea} and gp70^{env-sea} are indicated by arrows. (Left) Numbers indicate the position of molecular weight markers. In all cases, rabbit anti-gp^{env} serum was used for immune precipitation. (B) *ts sea* erythroblasts were grown at 37°C in CFU-E medium or induced to differentiate for 24 hr at 42°C, using EPO-containing differentiation medium (see Materials and methods). Cells were then labeled for 2 hr with [^{35}S]methionine (^{35}S Met) and immunoprecipitated subjected to the immune complex kinase assay (kinase) or labeled with [^{32}P]orthophosphate (^{32}P in vivo) immunoprecipitated. In all cases, rabbit antibody against bacterially expressed *v-sea* protein was used as immune serum (I), whereas normal rabbit serum was used in the controls (N). Positions of gp155^{env-sea}, gp70^{env-sea}, and molecular-weight markers are indicated as in A.

pressed at the cell surface in both fibroblasts and erythroblasts after shift to the nonpermissive temperature. However, although surface expression of gp155/70^{env-sea} remained unchanged in fibroblasts after shift to 42°C (Fig. 4B), a gradual decrease in its surface expression was seen in *ts1* S13 erythroblasts induced to differentiate at 42°C (Fig. 4A). This is most likely due to the observed down-regulation of gp155/70^{env-sea} biosynthesis in maturing erythroid cells (data not shown), which probably occurs in parallel to the general down-regulation of erythroblast glycoproteins seen previously in differentiating erythroblasts (Beug and Hayman 1984).

Erythrocyte-specific gene expression in immature, self-renewing ts1 S13-erythroblasts before and after temperature-induced differentiation

Characterization of purified, erythroblast populations transformed by *ts1* S13 or *wt* S13 revealed that they represent immature, self-renewing cells with many properties of the late erythroid progenitor (CFU-E). During temperature-induced differentiation, they cease to express several erythroblast antigens detected by monoclonal antibodies and polyclonal antisera while starting to express several differentiation antigens characteristic

Figure 4. Cell-surface expression of the S13-transforming proteins is not thermosensitive. *ts1* S13 (*ts sea*)-transformed erythroblasts (A) and fibroblasts (B) were grown at 37°C or shifted to 42°C for the times indicated. Cells were then stained with an absorbed tumor-bearing rat antiserum to gp155/70^{env-sea} (Hayman et al. 1985) in indirect immunofluorescence. The specificity of staining is indicated by the fact that producer erythroblasts transformed by AEV virus (AEV-*eb1*) are completely negative (see inset).

of erythrocytes (Schmidt et al. 1986a,b). It remained unclear, however, whether the immature cells could tolerate the expression of erythrocyte-specific proteins and how the activity of the respective genes would change during erythroid differentiation. In a first series of experiments, purified immature *ts1* S13 erythroblasts maintained at 37°C or shifted to 42°C for 2 days were analyzed by indirect immunofluorescence for expression of four red cell cytoskeleton proteins, namely anion transporter (band 3), band 4.1, spectrin, and an approximately 200-kD protein recognized by the MC2.7.3 monoclonal antibody, probably representing ankyrin. This technique allows the detection of these proteins at the single cell level. Figure 5 clearly shows that most cells in the population maintained at 37°C already expressed all four proteins at low but clearly detectable levels. Upon shift to 42°C, expression of band 3 and the MC2.7.3 antigen was clearly increased in essentially all cells of the population, whereas no major changes in expression were seen for band 4.1 and spectrin with this technique.

These results, showing that our cell populations were essentially homogeneous with respect to expression of the erythrocyte-specific proteins tested, encouraged us to study expression of erythrocyte-specific proteins at the biochemical level, using large-scale preparations of purified uninduced and temperature-induced *ts1* S13 cells similar to those shown in Figure 5. Aliquots of

these cell preparations were then analyzed for protein expression and biosynthesis by Western blotting and immunoprecipitation of [³⁵S]methionine-labeled cell extracts, as well as for mRNA expression by Northern blotting. Figure 6A demonstrates that the immature cells kept at 37°C synthesized band 3, band 4.1, and globin, whereas no MC2.7.3 antigen was seen (perhaps because most of the biosynthesized protein was rapidly degraded (Woods et al. 1986). After shift to 42°C, globin, band 3, and the MC2.7.3 antigen were produced at highly increased rates, whereas no such increase was seen for band 4.1 (Fig. 6A) and spectrin (data not shown). However, the pattern of band 4.1 polypeptide synthesis changed from an immature pattern (as seen in early embryonic erythroblasts) to a mature pattern (Staufenbiel and Lazarides 1986).

A similar result (increased expression of band 3 but not of band 4.1 and spectrin) was also obtained after Western blotting of total cell extracts (Fig. 6B). However, the pattern of band-4.1 proteins present in the temperature-induced cells did not yet correspond to that found in mature erythrocytes, suggesting that 48 hours of differentiation induction were sufficient for a complete 'switch' of the biosynthetic program but not for complete replacement of immature with mature-band 4.1 polypeptides.

Figure 5. Detection of red cell skeleton proteins in immature, self-renewing and temperature-induced *ts1* S13 erythroblasts by immunofluorescence. *ts1* S13 erythroblasts (clone A4) were grown at 37°C or shifted to 42°C for 48 hr (insets) and purified and dense cytopins were prepared (see Materials and methods). The dried cytopins were fixed, permeabilized, and stained with monoclonal antibodies to anion transporter (band 3), band 4.1, and the MC2.7.3 antigen, as well as with a polyclonal rabbit anti- α - β -spectrin antibody. (Top) The same fields of cells double stained with monoclonal anti-band 3 antibody and anti-spectrin antibody, respectively, followed by incubation with the respective fluorescein- and rhodamine-labeled second antibodies (see Beug and Hayman 1984; Materials and methods).

Figure 6. Expression of erythrocyte-specific proteins and their mRNAs in immature and temperature-induced *ts1* S13 cells (A) *ts1* S13 erythroblasts (clone A4) were grown at 37°C or shifted to 42°C for 2 days. After purification, cells were labeled with [³⁵S]methionine and subjected to immunoprecipitation analysis with monoclonal antibodies to α - β -globin, anion transporter (band 3), band 4.1, and MC2.7.3 (see Materials and methods). Fluorographs of SDS-polyacrylamide gradient gels were exposed for 1 day (globin), 4 days (band 3), and 12 days (MC2.7.3, band 4.1). Note the different variants of the band-4.1 protein occurring in immature and differentiated cells (Staufenbiel and Lazarides 1986). (B) Uninduced and temperature-induced *ts1* S13 (S13) cells were lysed in SDS-PAGE sample buffer and subjected to Western blot analysis as described in Materials and methods. As controls, purified preparations of red cell membranes (RSK) were run in parallel. (C) Total RNA was extracted from 4×10^7 to 6×10^7 uninduced and temperature-induced *ts1* S13 cells prepared as in A. After electrophoresis in agarose gels (10 μ g RNA/lane) and blotting to gene screen membrane, RNAs were hybridized to labeled probes specific for the genes indicated (see Materials and methods). Blots were exposed to film for 6 hr (band 3), 16 hr (band 4.1), 16 hr (Ala S), and 2 hr (α - and β -globin).

The protein data indicating that immature, self-renewing *ts1* S13 erythroblasts already express a variety of erythrocyte-specific genes at relatively low level, some of which are markedly up-regulated during temperature-induced differentiation, were confirmed and extended by Northern blot analysis of total RNA. *ts1* S13 cells kept at 37°C expressed detectable levels of mRNA specific for α - and β -globin, band 3, Na⁺K⁺ ATPase, band 4.1, β -actin and Ala S (δ -aminolevulinic acid synthetase, a key enzyme in heme biosynthesis). Upon shift to 42°C, steady-state levels of mRNA for both globins, anion transporter, and Ala S showed a marked increase, whereas no such changes were seen for Na⁺K⁺ ATPase, band 4.1, and β -actin. (Fig. 6C and data not shown). Nuclear run-on analysis confirmed that up-regulation of the

globin, anion transporter, and Ala S genes occurred at the transcriptional level (data not shown).

The results shown so far do not resolve the question of whether the observed low-level expression of erythrocyte-specific genes in transformed, self-renewing *ts1* S13 erythroblasts is a trait of normal erythroid progenitors or an aberrant phenomenon due to the transformed state of the cells. Because normal erythroid progenitors in numbers sufficient for such studies cannot be isolated, we addressed this question in an indirect manner. Based on the assumption (found to be true for other temperature-sensitive kinase oncogenes such as *ts src*) that the gp155/70^{env-sea} associated kinase activity in *ts1* S13 erythroblasts is destroyed within a few hours after shift to 42°C, we analyzed these cells for early changes in erythrocyte gene expression, which would precede up-regulation of gene expression due to the onset of differentiation. The results shown in Figure 7 clearly show that low-level expression of mRNA for β -globin, band 3, and Ala S persisted during the first 5 hours after shift, although some reduction in expression level was visible for Ala S and perhaps band-3 mRNA. In contrast, expression of mRNA for carbonic anhydrase (expression of which is only turned on late during mouse erythropoiesis; Conscience et al. 1987) was down-regulated to very low levels within 5 hours, indicating that this gene is

Figure 7. Kinetics of erythrocyte-specific RNA expression shortly after shift to 42°C. *ts1* S13 erythroblasts were grown at 37°C or shifted to 42°C for 2.5 hr (2.5 h), 5 hr, 10 hr, or 2 days. Extraction of RNA and Northern blot analysis was performed as described in the legend to Fig. 6. Exposure of the film showing the carbonic anhydrase RNA was for 5 days.

aberrantly expressed in the transformed cells and confirming that the cells 'normalize' to some extent before up-regulation of gene expression due to maturation, which occurred after 10 hours for globin, band 3, and Ala S and only after 48 hours for carbonic anhydrase. Taken together, these data confirm our earlier notion, that *ts1 S13* erythroblasts resemble normal erythroid progenitors in many respects, but in addition, exhibit aberrant features, such as elevated or even asynchronous expression of erythroid differentiation markers (Schmidt et al. 1986a,b). Upon shift to 42°C, however, these aberrant features are rapidly lost, suggesting that erythroid differentiation in the temperature-sensitive *S13* system starts from essentially normal progenitor like cells.

Discussion

The thermosensitive lesion in gp155/70^{env-sea} affects its autophosphorylation on tyrosine in vitro and in vivo

In this paper we have described the isolation and characterization of a mutant of the *S13* virus (*ts1 S13*) that is temperature sensitive for transformation of both fibroblasts and erythroblasts. Our results demonstrate that the temperature-sensitive lesion in the transforming protein of this mutant (gp155/70^{env-sea}) affects its kinase activity, the autophosphorylation of this protein being thermolabile both in an in vitro immune-complex assay and after ³²P-labeling of intact cells. However, neither cleavage of the gp155^{env-sea} precursor into gp70^{env-sea} and gp85^{env} nor its maturation and insertion into the plasma membrane were grossly affected by the temperature-sensitive lesion. Thus, the *ts1 S13*-transforming protein represents a thermolabile tyrosine kinase similar to those found in temperature-sensitive-*v-src* and temperature-sensitive *v-fps* mutants of Rous sarcoma virus (RSV) and Fujinami sarcoma virus (FSV). The temperature-sensitive *sea* protein, however, is distinct from the temperature-sensitive-*erbB* proteins of *ts34* and *ts167* AEV (Graf et al. 1978; Palmieri et al. 1982; Beug and Hayman 1984); the temperature-sensitive lesion of which affects glycoprotein maturation and plasma membrane localization but not its associated kinase activity (Hayman et al. 1986).

Although our data indicate that the tyrosine kinase activity of the *env-sea* protein of *S13* virus is required for transformation, it remains an open question whether or not both forms of this protein, gp155^{env-sea} and gp70^{env-sea}, are transforming.

Temperature-sensitive sea-transformed erythroblasts: An improved cell system to study erythroid differentiation

Although erythroblasts transformed by the available temperature-sensitive AEV mutants have proved a valuable system to study erythroid differentiation, the *ts1 S13*-transformed erythroblasts described here appear to be far better suited to mimic the differentiation of normal erythroid progenitors because of two recent

findings. First, the *v-erbA* protein of temperature-sensitive AEV mutants, *v-erbA*, causes a transcriptional arrest of expression of several erythrocyte-specific genes, which can be partially overcome by use of special culture conditions (Beug et al. 1982; Beug and Hayman 1984; M. Zenke et al., in press). Furthermore, the *erbB* proteins of both *ts34* and *ts167* AEV display a distinct, residual transforming activity at nonpermissive temperature in both fibroblasts and erythroblasts (Beug and Graf 1980; Palmieri et al. 1982; H. Beug et al., unpubl.). Because it has been much easier to induce transformed erythroid colonies with *ts1 S13* than with the other available temperature-sensitive kinase mutants of RSV and FSV (Kahn et al. 1984; H. Beug, unpubl.), the latter viruses also do not seem to represent a reasonable alternative to *ts1 S13*.

As in any other in vitro cell system employing differentiation-inducible, but transformed erythroid cells, it is clear that differentiation in the temperature-sensitive *S13* cell system starts from self-renewing, partially aberrant erythroblasts rather than from normal erythroid progenitors that do not self-renew. Although this fact requires that any conclusions derived from the temperature-sensitive *S13* system can only be applied to normal erythropoiesis with caution, the available data suggest that due to the rapid and fairly complete thermal inactivation of the transforming oncogene, few if any traits of the transformed cell persist during temperature-induced differentiation of the *ts1 S13* erythroblasts.

Erythrocyte-specific gene expression during self-renewal and differentiation of ts1 S13-transformed erythroblasts

Leukemic transformation of avian erythroid cells by a variety of tyrosine kinase as well as non-kinase oncogenes, such as *v-H-ras*, is characterized by induction of continuous self-renewal of the infected, immature progenitor cells (Graf et al. 1976; Beug et al. 1985a). It was therefore surprising that these self-renewing, leukemic cells already expressed low levels of most if not all erythrocyte-specific proteins. This result suggests that the proliferation of transformed erythroid progenitors is compatible with low-level expression of several major erythrocyte proteins that are characteristic of the mature postmitotic erythrocyte. Therefore, induction of self-renewal by these oncogenes does not lead to a complete block of erythrocyte-specific gene expression but may only require that these genes are not expressed at levels exceeding those of the housekeeping proteins, which are characteristic of immature, proliferating cells. According to this concept, only quantitative (perhaps even small) changes in the relative rates of synthesis of these two types of molecules might be required to convert a normal, maturing progenitor into a self-renewing leukemic cell.

The second conclusion suggested by our studies is that some erythrocyte proteins such as the globins, anion transporter, Ala-S, carbonic anhydrase, and the MC2.7.3 antigen are drastically up-regulated relatively

late in erythroid differentiation, whereas the expression of other erythrocyte proteins, for example, band 4.1 and spectrin, is not controlled in this way. Alternatively, expression of the former proteins might already be high in the normal progenitors representing the target cells for leukemic transformation. If this is correct, the main effect of the transforming oncogene would be to reduce, but not to abolish, expression of certain erythrocyte-specific genes.

Recently, it has been suggested that erythroid differentiation is regulated by late, asynchronous expression of the anion transporter (Woods et al. 1986). However, the absence of anion transporter in AEV-transformed erythroblasts observed by Woods et al. appears to be due to the presence of the *erbA* oncogene in these cells (M. Zenke et al., unpubl.). The apparent discrepancy between our results on anion transporter expression in S13-transformed erythroblasts and those obtained by Woods et al. (1986) is not easily explained at present and will merit further experimentation. The data presented in this paper do not strongly support the idea of the anion transporter playing a crucial regulatory role in early stages of erythroid differentiation, as it is already expressed in immature erythroblasts. However, it is certainly possible that the anion transporter does play a pivotal role in the assembly of the erythrocyte differentiation as well as for structure and function of the mature red blood cell.

Outlook

The experiments described herein using *ts1* S13-transformed erythroblasts demonstrate the usefulness of this new system to study gene expression during erythroid differentiation. It was found that erythrocyte-specific genes are not expressed as a coordinate group of genes and that the expression of these gene products typical for mature, postmitotic cells is not incompatible with cell growth. The temporal regulation of genes such as band 3 and MC2.7.3 contrasts markedly with the regulation of expression of the various forms of the band-4.1 proteins and demonstrates the potential of this system for unraveling the complexities of cytoskeleton assembly and erythroid differentiation.

Materials and methods

Virus strains

The origin and characterization of the S13 virus has been described earlier (Benedict et al. 1985; Beug et al. 1985b). The *ts1* S13 virus substrain was isolated from a nonproducer erythroblast clone (clone 9; Beug et al. 1985b) superinfected with RAV-1, whereas the *wt* S13 strain has been cloned in nonproducer fibroblasts (P. Vogt, unpubl.) superinfected with RAV-1.

Cells and cell culture

ts1 S13 and *wt* S13 erythroblast clones were produced by seeding infected bone marrow cells into semisolid medium (Methocel) and isolation of transformed colonies 6–8 days later

(Beug et al. 1985b). Unless stated otherwise, all cell clones were propagated in CFU-E medium without anemic chicken serum (Radke et al. 1982; Beug and Hayman 1984). Well-differentiating *ts1* S13 erythroblast clones exhibiting long (>40 generations) *in vitro* life spans were selected from over 100 individual colonies, as described earlier (Beug and Hayman 1984; Kowenz et al. 1987).

CEFs transformed by *ts1* S13 and *wt* S13 viruses were produced by cocultivating CEFs freshly prepared from 11-day-old chick embryos (Graf 1973) with the respective virus-producing erythroblasts for 2 days (Beug et al. 1986). Both infected and uninfected fibroblasts were grown in standard growth medium (Dulbecco's modified Eagle's medium (DMEM) plus 8% fetal calf serum, 2% chicken serum, and 25 mM HEPES, pH7.3).

Large-scale temperature induction of erythroid differentiation in temperature-sensitive S13 erythroblasts

Prior to differentiation induction, *ts1* S13- and *wt* S13-transformed erythroblasts were separated from spontaneously differentiating cells by purification on Percoll step gradients (using only cells with a density of ≤ 1.070 g/cm³). The cells were then seeded at 1×10^6 to 1.5×10^6 cells into a modified differentiation medium (Kowenz et al. 1987) supplemented with 150 μ l EPO (high-titer anemic chicken serum prepared according to Kowenz et al. 1987), 5 μ l of partially purified erythroid growth factor produced by an avian reticuloendotheliosis virus (REV-T) transformed lymphoblast cell line (M. Zenke et al., in prep.), and 6 μ l insulin (1.7 mg/ml, Actrapid, Bayer, Leverkusen, Germany) per 10 ml of medium and incubated at 42°C and 5% CO₂. Cell density was kept between 1×10^6 to 2×10^6 cells/ml by addition of the above medium, and a partial medium change was performed daily. Because of the relative homogeneity of the differentiated cell populations, it was sufficient to remove dead or malformed cells by centrifugation through Ficoll (37°C and 12–24 hr at 42°C), Percoll 1.072 g/cm³ (48 hr at 42°C), or Percoll 1.080 g/cm³ (72 hr at 42°C; Beug et al. 1982; Schmidt et al. 1986b,c). Control cells grown at 37°C were purified on Percoll 1.070 g/cm³ as described above.

Assays for stimulation of proliferation and hemoglobin production of temperature-sensitive S13 cells by EPO

These assays have been described elsewhere (Kowenz et al. 1987).

Assays for fibroblast transformation parameters

ts1 S13- and *wt* S13-transformed fibroblasts were cultivated at 37°C or 42°C for at least 24 hr. They were then assayed for morphology, actin filament cables, fibronectin expression, and uptake of [³H]2-deoxyglucose, as described earlier (Royer-Pokora et al. 1978; Palmieri et al. 1982). Agar colony formation of virus-infected, primary fibroblasts was assayed as described elsewhere (Jansson et al. 1987).

Assay of differentiating ts1 S13 erythroblasts for morphology, hemoglobin, content, and size

Cytospin preparations of *ts1* S13 erythroblasts were stained with neutral benzidine plus histological dyes (Diff-Quik, Harleco, Amsterdam), as described earlier (Beug et al. 1982). Size distribution was measured using a Coulter counter connected to a multichannel analyzer (32 channels, constructed in the EMBL). Under the particular conditions used, chick erythrocytes (mean diam. 5 μ m) band at channel 4, whereas trans-

formed erythroblasts (mean diam. 12 μ m) band at channels 18–22.

Antisera

The following antibodies to erythrocyte-specific proteins were used: MC4.2.A.5, a monoclonal antibody reacting with embryonic and adult chicken hemoglobins (Hayman et al. 1982); MCA2E, a monoclonal antibody to the chicken anion transporter (Yew et al. 1987); MC3C, a monoclonal antibody to chicken band 4.1 (Yew et al. 1987); MC2.7.3, a monoclonal antibody directed against a ~200-kD red cell skeleton protein [a kind gift of H. Weintraub, Seattle]; and a polyclonal rabbit antiserum to α - β -spectrin (J.D. Engel et al., unpubl.). The ~200-kD protein detected by the MC2.7.3 antibody might represent ankyrin, because it is present in large amounts in purified red cell skeleton preparations but differs from α - β -spectrin both in mobility on gels and in its expression during temperature-induced differentiation (H. Beug and M.J. Hayman, unpubl.). Antisera to the gp155/70^{env-sea} protein of S13 virus were anti-gp^{env} serum (Hayman et al. 1979), tumor-bearing rat antisera (Hayman et al. 1985), and a rabbit antiserum to a v-sea polypeptide expressed in bacteria (J. Knight et al., unpubl.).

Immunofluorescence

Staining of live, transformed fibroblasts with a tumor-bearing rat antiserum detecting S13-specific *env* determinants at the cell surface was performed as described earlier (Hayman et al. 1985).

Dense cytospin preparations (3×10^5 to 4×10^5 cells/sample) were prepared as described earlier (Beug et al. 1982) and air dried. They were fixed in 3.7% formaldehyde at 4°C for 30 min, washed once each in PBS and DMEM plus 10% fetal calf serum and 25 mM HEPES (pH 7.3), permeabilized for 15 min in the above medium also containing 0.1% NP-40, and washed twice with phosphate-buffered saline (PBS) plus 0.2% gelatin and 0.5% bovine serum albumin (BSA). The fixed cytospin preparations were then stained with anti-erythrocyte cytoskeleton antibodies in double immunofluorescence, as described earlier (Beug et al. 1979; Beug and Hayman 1984), using fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Fab₂ (Medac, Hamburg, Germany, 1:30) and Texas-red conjugated goat anti-rabbit IgG (Amersham-Buchler, 1:30).

Immunoprecipitation analysis

Labeling of purified cell populations with [³⁵S]methionine and immunoprecipitation analysis was done as described earlier (Hayman et al. 1983; Beug and Hayman 1984), except for the following modifications: For immunoprecipitation of gp155/70^{env-sea}, globin, and band 3, cells were lysed in detergent-containing lysis buffer as described (Hayman et al. 1979, 1983). Immunoprecipitation of the MC2.7.3 antigen and band 4.1 was performed after lysis of 10×10^6 cells in 0.1 ml 2% SDS (15' 37°C), dilution with 1.9 ml of the above lysis buffer, and centrifugation (20,000g, 30 min). For immunoprecipitation with monoclonal antibodies, the *Staphylococcus aureus* preparations used to collect the immune complexes were preincubated for 30 min with 10 μ g of affinity-purified rabbit anti-mouse IgG per 30 μ l of 10% Staph A suspension. Immunoprecipitated proteins were analyzed by SDS-polyacrylamide electrophoresis and fluorography (Beug et al. 1981; Beug and Hayman 1984), using 6–12% gradient gels for all proteins except globin, where 6–20% gradient gels were used.

Western blotting analysis

Analysis of red cell cytoskeleton proteins was performed as described elsewhere (Leutz et al. 1987), using purified avian red cell membranes (D. Engel, unpubl.) as standards. Briefly, cells were lysed in SDS-PAGE sample buffer at 1×10^8 cells/ml and equal aliquots run on a 6–12% denaturing gel. Proteins were transferred to nylon membranes (BioRad) and reacted with antibodies plus ¹²⁵I-labeled protein A as described in Leutz et al. (1987). When monoclonal antibodies were used, the membranes were reacted with affinity-purified rabbit anti-mouse IgG antibody prior to application of ¹²⁵I-labeled protein A.

³²P labeling of S13-transformed cells and in vitro tyrosine kinase assay of gp155/70^{env-sea}

The in vitro immune complex kinase assays were performed as described elsewhere (Hayman et al. 1985, 1986). For labeling with ³²P-labeled PO₄, approximately 2×10^7 cells were labeled for 4 hr with 1 mCi [³²P]orthophosphate per ml of phosphate-free DMEM supplemented with 2% dialyzed fetal calf serum. The cells were then lysed in 10 mM HEPES (pH 7.3), 10% glycerol, 150 mM NaCl, 50 μ M Na orthovanadate, and 0.1% Triton X-100 and immunoprecipitated as described above.

For quantitative evaluation, the gel autoradiographs were scanned, and the peak area determined by cutting out the peaks from the paper strip and weighing, using a microbalance. Because of the relatively high background of the gels (Fig. 3B,C), lower limits of the differences (>20-fold) are given in the text rather than the exact figures.

RNA preparation and Northern blot analysis

Total RNA has been prepared using the guanidinium thiocyanate/CsCl step gradient procedure (Glisin et al. 1974; Maniatis et al. 1982) with the modification that a mixture of 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 1% sarkosyl was used to dissolve the RNA after CsCl step gradient centrifugation and for subsequent extractions.

After gel electrophoresis (1% agarose) in 0.2 M formaldehyde (Lehrach et al. 1977; Maniatis et al. 1982) the RNA (10–20 μ g) was blotted to GeneScreen membrane (NEN) in 20 $\times$ SSC and cross-linked by UV treatment. Hybridization with α [³²P]dCTP-labeled probes, prepared by nick-translation (Rigby et al. 1977), was performed in 45% (vol/vol) formamide, 0.2 M Na phosphate (pH 7.2), 1 mM EDTA, 7% SDS, 100 μ g/ml denatured salmon sperm DNA, 10⁶ cpm/ml at 42–45°C for 16 hr. The membranes were subsequently washed in 40 mM Na phosphate (pH 7.2) and 1% SDS at 55–65°C for 1–2 hr. The recombinant DNAs used for probe preparation by nick-translation were as follows: (band 3) pcB3 2.0 and pcB3 3.1 containing 2.0 and 3.1 kb, respectively, of chicken erythroid band 3-specific cDNA sequences (M. Zenke et al., in prep.); (band 4.1) p4.1A, bearing 1.9 kb of chicken band 4.1 cDNA in pGEM2 (Yew et al. 1987); (β -globin) p1BR15, containing the entire chicken β -globin gene (Dolan et al. 1983); (β -actin) pA13, which contains 2 kb of chicken cytoplasmic β -actin cDNA in the *Hind*III site of pSP64 (Cleveland et al. 1980); (carbonic acid anhydrase) pCA-1.2, containing 1.2 kb of chicken carbonic anhydrase II cDNA (Yoshihara et al. 1987).

The Ala S probe used was an α -[³²P]GTP-labeled transcript from the SP6 promoter of recombinant pA4d, containing a 775-bp cDNA insert in the *Eco*RI site of pGEM1 (Yamamoto et al. 1985). Hybridization conditions were the same as for nick-translated probes, except that the hybridization temperature was 65°C. After hybridization, the membrane was treated with

RNase A (5 µg/ml) in $2\times$ SSC at 37°C for 30 min following washing, as described above.

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