To the Editor:

The recent report by Koury et al on the localization of erythropoietin (EPO)-producing cells in the murine liver brings to light the ongoing difference of opinions in the interpretation of results following in situ hybridization. Although the beauty of the in situ hybridization procedure is that one can detect gene expression on a per-cell basis, it is a highly subjective method. Furthermore, a balance has to be found between signal resolution and cellular morphology. Unfortunately, like many techniques, in situ hybridization can also be plagued with problems, which may not at first be apparent. One such problem appears to have occurred not only in the detection of EPO gene expression in the liver, but also in the kidney.6 Besides an extremely high background, the concentration of signal is found over the nucleus and not, as would be expected, over the cytoplasm. The same phenomenon appears to be the case, but to a much lesser extent, in unbleached transgenic control mice. In the most recent report,7 the investigators themselves state that they are unsure as to why there should be a preferential concentration of silver grains associated with the nucleus and perinuclear region for both transgenic and nontransgenic mice. Is this not a cause for concern? Perhaps some may view this as making a mountain out of a molehill. Certainly, some radioactive tracks emanating from a source near the nucleus could end up as a latent image over the nucleus. However, in the present case it appears that the source of the latent image is the nucleus and not the cytoplasm. One could argue that because a signal is not seen over all cells, specific hybridization has taken place. However, this should not be assumed and need not necessarily be the case.8

The question is: why is the radioactive signal concentrated over the nucleus? I would like to suggest a reason for this by drawing attention to a phenomenon called positive chemography.9 The latter is the spontaneous autoradiographic activation of silver grains producing a latent image. Exactly how positive chemography occurs is not at first apparent. One such problem appears to have occurred not only in the detection of EPO gene expression in the liver, but also in the kidney.9 Furthermore, the presence in the in situ hybridization buffer of reducing agents such as β-mercaptoethanol, which has been used by the investigators,10 or dithiothreitol, could also enhance the positive chemographic artifact. The latter is usually observed as a local aggregation of silver particles formed in a single plane (K.-H. Halbhuber, University of Jena, personal communication, July 1991).

Koury et al suggest that by using 35S-labeled probes with thinner sections and emulsions the resolving power can be optimized. There is no doubt that the cells can be clearly seen even at high magnification. However, although the usual controls for in situ hybridization appear to have been performed, controls for autoradiographic procedure, chemography in particular are missing. This, together with the fact that the signal is not where it should be, raises considerable doubt as to whether the cells claimed to produce EPO are actually doing so. Considering the intensity of the signals obtained, it should be an easy exercise to substantiate EPO production by the immunohistochemical detection of intracellular EPO. It is very unlikely that in both the liver and kidney EPO is being secreted at such a rapid rate that it cannot be detected by a specific antibody. Therefore, unless the investigators can demonstrate that no artifacts have occurred or they can explain these extraordinary findings, I for one am not yet willing to accept the proposed localization of EPO gene expression in either the liver or the kidney.

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Dr Rich states that in situ hybridization is a “highly subjective” method. When correctly performed and properly controlled, in situ hybridization is, in fact, an objective method. Based on the controls that we have performed, Dr Rich is incorrect in his assertion that “positive chemography” is the cause of silver grains over interstitial cells in the kidney and both hepatocytes and nonhepatocytes in murine liver. If chemography were in fact the cause of the labeled cells in the emulsions, such cells would be present in equal numbers in anemic and nonanemic kidneys and livers, which is clearly not the case. One might argue that the tissue hypoxia which results from making a mouse anemic could cause chemical changes in the nuclei that would lead to chemography artifacts, but we have controlled for that possibility as well. Because we used single-stranded RNA probes in our studies, we performed hybridizations with antisense and with sense strands. Only in autorigraphs from tissues hybridized with antisense RNA probes did we see cells in the kidney or liver overlaid with silver grains. If “positive chemography” was involved in generating the autoradiographic signal, then the signal would have also been present when the sense strands were used in the in situ hybridizations. We have stated clearly in our recent reports that both sense and antisense strands were used to perform the in situ hybridizations and that autoradiographic signals were only seen with antisense probes. In our most recent publication, we also found that the human erythropoietin (EPO) cRNA probe used in that study hybridized to hepatocytes in the livers of transgenic mice that exhibit liver-specific expression of the human EPO transgene. The human EPO probe did not hybridize to kidneys of the same mice (where the human transgene was not expressed), even though the mouse antisense EPO RNA probe did hybridize to interstitial cells in these same transgenic kidneys in the usual fashion. If “positive chemography” were responsible for the hybridization signal seen with the mouse probe, then we should have seen hybridization with the human probe as well. Furthermore, Semenza et al have recently found that in lines of transgenic mice which do exhibit inducible expression of the human EPO transgene in the kidney as well as liver, the human EPO cRNA probe hybridizes only to interstitial cells in the kidney. The human probe hybridizes strongly to such cells in anemic mice of these lines and mimics completely the observations obtained with the mouse probes in both nontransgenic and transgenic mice.

There are several other lines of evidence that support our conclusions as to the types of cells that produce EPO. Lacome et al found interstitial cells also hybridized with their EPO cDNA probes. Schuster et al have recently cloned a rat EPO cDNA, which hybridizes to interstitial cells in the kidney and to both hepatocytes and nonhepatocytes in liver when used for in situ hybridizations in rat kidney and liver. Thus, several different probes, labeled in different ways and used for in situ hybridizations in different species, consistently identify renal interstitial cells as the cells that produce EPO in the kidney. Furthermore, we have presented data in which we correlated the number of EPO-producing renal interstitial cells with kidney EPO mRNA levels and serum EPO concentrations at three different levels of acute anemia and during the recovery from an acute anemia. The number of EPO-producing cells correlated extremely well with the amount of EPO mRNA present in the kidneys of mice with similar hematocrits under all conditions tested. We think all of these observations indicate that specific hybridizations of our EPO probes are responsible for the silver grains in our autoradiograms.

Dr Rich questions why the silver grains in our autoradiograms are associated with the nucleus of EPO-producing cells in the kidney and liver. The renal interstitial cells and the nonhepatic EPO-producing cells in the liver have only thin rims of cytoplasm surrounding their nuclei. Because the cytoplasm of these cells is so scarce, it is impossible for the grains to be associated with anything but the nucleus. Dr Rich expects that the silver grains over hepatocytes should be more evenly distributed over the abundant cytoplasm of these cells. We do not know precisely what the subcellular localization of EPO mRNA is in hepatocytes, but we do know that the silver grains in our autoradiograms are associated with the nucleus and perinuclear region. Dr Rich states that we have an “extremely high background” in our autoradiograms. In our initial studies, the background was higher than we would consider acceptable at the present time. However, in those early studies the signal-to-noise ratio was sufficient as to allow us to detect interstitial cells as the only source of EPO in the kidney. Dr Rich has certainly been to meetings where our more recent in situ hybridization work has been presented and should also have seen recent reviews containing photomicrographs of kidney autoradiograms in which background is negligible. The background in nontransgenic mouse liver is higher than that in kidney due to the longer exposure times required to generate a signal.

Dr Rich also suggests that we substantiate EPO production in the kidney and liver through the use of immunohistochemistry. Several groups have used immunohistochemistry to localize EPO-producing cells within the kidney and all have found different locations. The problem with immunohistochemical detection of EPO in the kidney is that because EPO circulates in plasma and is excreted through the kidney, one cannot distinguish between production, filtration, or nonspecific adherence of EPO at any given site. The reason we chose in situ hybridization to localize EPO-producing cells was to avoid this ambiguity. It is our belief that detection of EPO mRNA in cells is the strongest criterion that we can apply for determining which cells produce EPO. It is formally possible that a cell type could produce EPO transcripts that are nonfunctional. However, we have never detected evidence for any other cell type producing EPO mRNA.

Dr Rich is a proponent of the theory that tubule cells are responsible for EPO production in the kidney as put forth by Maxwell et al. Dr Rich has presented work in abstract form that he believes corroborates the work of Maxwell et al, and has used in situ hybridization in an attempt to demonstrate that a subpopulation of macrophages produces EPO. It should be pointed out that Dr Rich has never demonstrated the specificity of his biotin-labeled EPO DNA probe by anything other than a dot-blot and he uses a detection system (reflection contrast microscopy) not commonly used by others for in situ hybridization studies.

In the in situ hybridization study mentioned above, Maxwell et al used a 32P-labeled oligonucleotide probe, 10-μm frozen sections, and a thick emulsion. Such conditions make it highly unlikely that the investigators could resolve EPO production at the cellular level, i.e., the oligonucleotide probe might be specifically hybridized to EPO mRNA, but the grains in the autoradiogram may be due to microns away from the source due to the high energy of the β particles emitted by 32P. This may in fact be the case because the general pattern of scattered positive cells in their low-magnification photomicrographs is remarkably similar to that which we achieve in our severely anemic mice. However, on closer inspection of the higher magnification photomicrograph in their report, the autoradiographic signals are scattered over a variety of cell types.
The cell types include interstitial and possibly even glomerular cells well as the tubule cells claimed by the investigators.\textsuperscript{14} We chose to use a thinner emulsion and lower energy \textsuperscript{35}S-labeled probes, coupled with the superior morphologic detail obtained with paraformaldehyde-fixed, paraffin-embedded tissue cut at section thicknesses of 3 to 5 \si{\mu m} in our in situ hybridization studies. We believe that the increased ability to resolve the cellular source of the radioactive signal afforded by these conditions makes our interpretation of cortical interstitial cells as the source of EPO in the kidney the correct one. Similar arguments can be extended to the cells producing EPO in the liver as well.

\textbf{REFERENCES}

The site of erythropoietin production: localization of erythropoietin mRNA by radioactive in situ hybridization [letter; comment]

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