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A simple method for quantifying specific mRNAs in small numbers of early mouse embryos

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ABSTRACT

Amplification of sequences by the polymerase chain reaction (PCR) has become a powerful tool in the study of gene expression. The technique is, in fact, so powerful that it may detect 'leaky transcription'. Thus, it is now important to be able to quantify the transcripts that are amplified to determine whether or not they represent legitimate transcription of target genes. In this paper, we describe a one-step amplification reaction coupled to solution hybridization/RNase protection that is capable of quantitating specific transcripts in total RNA from one to ten preimplantation mouse embryos and is generally applicable to any cloned mRNA sequence.

INTRODUCTION

The polymerase chain reaction has been used to detect specific messages in preimplantation mouse and human embryos (1, 2, 3). This technique is so sensitive that sequences from a single embryonic blastomere can be amplified and detected (2, 3, 4). It has also been observed, however, that tissue-specific messages can be amplified and detected in reactions containing cDNA from so-called 'non-expressing' tissues (5, 6). Therefore, the ability to quantitate the amount of message present in a particular tissue is important. In this paper we describe a quick and easy method to reliably quantitate levels of both rare and abundant messages in total RNA from as few as one to ten preimplantation mouse embryos.

MATERIALS AND METHODS

RNA isolation

Total RNA was isolated from 3.5 day mouse blastocysts and from the livers of adult female mice by the acid guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (7). Isolation of total RNA from 3.5 day blastocysts was done in the presence of 100 μg of yeast tRNA, which was used as carrier. The percent recovery of blastocyst RNA was determined in several separate experiments in which known quantities of ^{35}S-labeled RNA were added with the carrier RNA to varying numbers of embryos (from 5 to 70) and the RNA isolated. The percent recovery of the tracer RNA ranged from 56% to 72%, with an average of 64%.

Oligodeoxynucleotides

The oligo (dT) primer used for first strand cDNA synthesis, $pd(T)_{12-18}$(5'-PO_4, Na^+ salt), was purchased from Pharmacia. The gene-specific primers used for amplification by the polymerase chain reaction were synthesized in-house. The β-actin 5' primer was 5'- TAAAGACCTCTATGCCAACACAGT-3', the 3' primer was 5'-CACGATGGAGGGGCCGGACTCATC-3' and the expected size of the amplified fragment was 240 nucleotides (n). The Hox 2.1 5' primer was 5'-CCCGGCGGCG-ACGTATCGAGATCG-3', the 3' primer was 5'-GCTCC-TCCGGATGGGCTCAAGGTT-3' and the expected size of the amplified fragment was 167 n.

Reverse transcription (two-step reactions)

The procedure followed for cDNA synthesis was only slightly modified from that of Gerard (8). 1 μg of total RNA isolated from liver and 5 μg of *Escherichia coli* rRNA (used as carrier) were heated to 70°C for 5 min and cooled on ice. The RNAs were mixed, in a final volume of 30 μl, with 1.5 μg oligo (dT), 250 μM each dNTP and 300 units of Moloney murine leukemia virus (MMLV) reverse transcriptase in 1×reverse transcription (RT) buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT). The mixtures were incubated at 37°C for 1 hr and the reactions stopped by the addition of EDTA to 30 mM. The cDNA was purified by phenol extraction, precipitated by ethanol and resuspended in water.

PCR amplification (two-step reactions)

One-tenth of the product cDNA (synthesis described above) was combined with 1 μg of each β-actin PCR primer, 150 μM each dNTP and 1 unit Taq DNA polymerase (Perkin Elmer Cetus) in 1×PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 100 μg/ml BSA) in a total volume of 50 μl. Each PCR cycle consisted of a denaturation step (95°C, 1 min), an annealing step (45°C, 2 min) and an elongation step (72°C, 2 min). For the first cycle only, the duration of the denaturation

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step was 2 min and for the final cycle only, the duration of the elongation step was 10 min. DNA was amplified for 60 cycles in a Coy Tempcycler.

RT-PCR (one-step amplification reactions)

1 μg of total RNA from mouse liver or varying amounts of synthetic RNA (see below) or blastocyst RNA were mixed with 5 μg *E. coli* rRNA and 1 μg each of the gene-specific 5′ and 3′ PCR primers. Yeast tRNA (equivalent to the amount of blastocyst total RNA used in each experiment) was added to reaction tubes containing synthetic message. The RNA/oligonucleotide mixtures were heated to 70°C for 5 min and cooled on ice. Each tube then received reaction mix, which contained 150 μM each dNTP, 300 units MMLV reverse transcriptase and 1 unit Taq DNA polymerase in 1×RT buffer (see above). In one case (Figure 1, lane b) 1×PCR buffer (see above) was used instead of 1×RT buffer. Final reaction volumes were 50 μl. The samples were incubated at 37°C for 1 hr in the Tempcycler to allow reverse transcription to occur, and then this program was immediately looped into the PCR amplification cycle described above.

Transcription of synthetic messages and riboprobes

The plasmid pGem1-act1 (a gift from M.Rogers and L.Gudas) contains approximately 750 bp of mouse β-actin cDNA subcloned into the *Pst*I site of the vector pGem1. The insert consists of the last 517 bp of coding sequence, 100−150 bp of the 3′ untranslated region and linker sequences. To transcribe synthetic β-actin message, plasmid pGem1-act1 was linearized with *Hin*dIII and transcribed with T7 RNA polymerase, in the presence of ^{32}P-UTP (800 Ci/mmol, NEN), to a specific activity of 1×10^6 dpm/μg. To transcribe anti-sense riboprobe, this plasmid was linearized with *Eco*RI and transcribed with SP6 RNA polymerase, in the presence of ^{32}P-UTP, to a specific activity of 5.2×10^7 dpm/μg.

The plasmid pGem3 H2.1/*Hae-Hae* 250 (a gift from M.Utset and F.Ruddle) contains a 240 bp *Hae*III genomic DNA fragment of the mouse Hox 2.1 gene cloned into the *Sal*I site of the vector pGem3. The insert consists of the last 124 bp of the homeobox plus 72 bp of 3′ flanking region. To transcribe synthetic Hox 2.1 message, plasmid pGem3 H2.1/*Hae-Hae* 250 was linearized with *Bam*HI and transcribed with T7 RNA polymerase, in the presence of ^{32}P-UTP, to a specific activity of about 1×10^6 dpm/μg. To transcribe anti-sense riboprobe, this plasmid was linearized with *Eco*RI and transcribed with SP6 RNA polymerase, in the presence of ^{32}P-UTP, to a specific activity of about 5.2×10^7 dpm/μg.

Transcription reactions contained 0.5 μg linearized plasmid DNA template, 500 μM each ATP, GTP, CTP, either 12.2 μM (for synthetic messages) or 15 μM (for riboprobes) UTP, either 0.1 μCi (for synthetic messages) or 10 μCi (for riboprobes) ^{32}P-UTP (800 Ci/mmol, NEN), 16 units RNasin, and 10 units of either T7 (for synthetic messages) or SP6 (for riboprobes) RNA polymerase in 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 10 mM dithiothreitol, 6 mM $MgCl_2$, 2 mM spermidine, and 50 μM EDTA. Reactions were performed in a final volume of 20 μl at 37°C (synthetic message, T7 polymerase) or 40°C (riboprobe, SP6 polymerase) for 1 hr. Template DNA was removed by digestion with 1 unit of RNase-free DNase (Promega) at 37°C for 15 min. The products were purified by extraction with phenol/chloroform and unincorporated nucleotides were removed by centrifugation through a 1 ml bed of Sephadex G-50 resin.

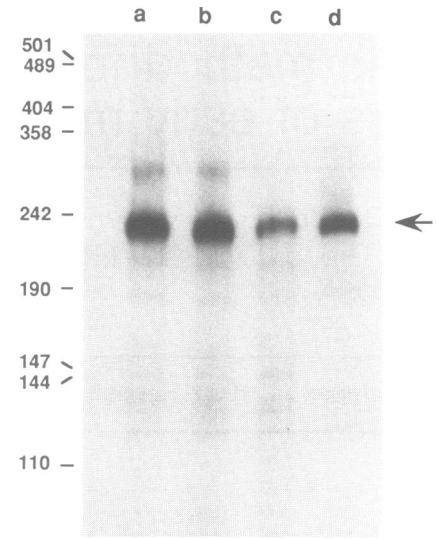

Figure 1. Comparison of one-step and two-step amplification reactions. β-actin transcripts in total RNA from livers of adult mice were amplified in either one-step (Lanes a and b) or two-step (Lanes c and d) reactions and 20% of the reaction volume was hybridized to labeled antisense β-actin riboprobe and subjected to RNase protection analysis. Lane a, one-step amplification of 1 μg liver RNA in 1×RT buffer; lane b, the same reaction in 1×PCR buffer. Lane c, two-step amplification of 100 ng liver RNA in the presence of MMLV reverse transcriptase; lane d, two-step amplification of 100 ng of liver RNA without MMLV reverse transcriptase. Autoradiographic exposure was for 5 min at 22°C.

The RNA was ethanol precipitated in the presence of 5 μg yeast tRNA (used as carrier).

To be certain that no contaminating template DNA remained after transcription and isolation of the synthetic message, a mock transcription reaction was digested with DNase as above for 0, 15 and 25 min and one-half of the reaction was subjected to electrophoresis through agarose. No template DNA was detected after either 15 or 25 min digestion when assayed by ethidium bromide staining or by hybridization to labelled probe after Southern transfer. Furthermore, the close agreement between the number of β-actin mRNA molecules per embryo determined by this method (see below) or by an entirely different method (9) also argues against plasmid DNA contamination.

To quantify the synthetic message produced, the exact specific activity of the ^{32}P-labelled nucleotide was calculated for the day used, following the equations and decay tables provided by the manufacturer and this nucleotide was assumed to be one-fourth of the total nucleotides incorporated into RNA. By using the specific activity of the synthesized RNA, calculated in this way, and by determining the total radioactivity incorporated into trichloroacetic acid precipitable material, it was possible to calculate precisely the total amount of RNA synthesized.

RNase protection analysis

PCR-amplified DNA was purified by extraction with phenol/chloroform and precipitated by ethanol. The entire amplification reaction (except for the preliminary experiment shown in Figure 1, for which 20% of the amplification reaction was used) was hybridized to a ^{32}P-labeled gene-specific riboprobe at 50°C for 16 hr in 80% formamide, 40 mM PIPES (pH 6.7), 0.4 M NaCl, 1 mM EDTA. Non-hybridized riboprobe was digested by 40 μg/ml RNase A and 2.2 μg/ml RNase T_1 in 10 mM Tris-HCl (pH 8.0), 5 mM EDTA and 300 mM NaCl

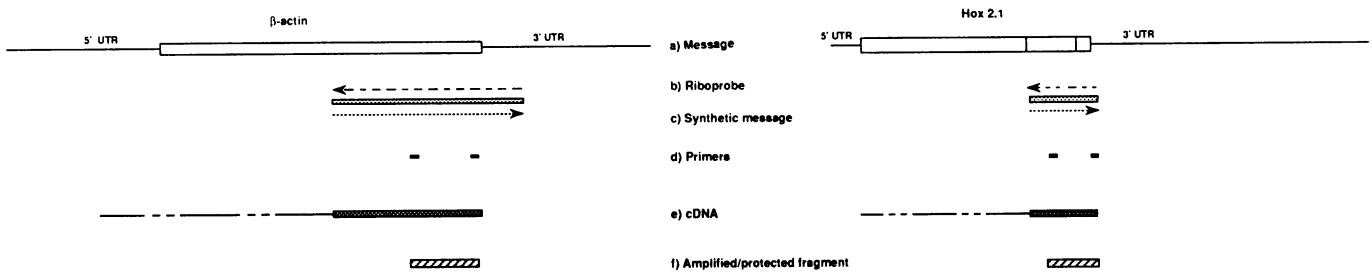

Figure 2. Schematic representation of the nucleic acid components of one-step amplification reactions.

a) messenger RNA	——	5′ and 3′ untranslation regions
	▭	coding sequence
	▭	homeobox-Hox 2.1 only
b) riboprobe/	▨	cloned region
c) synthetic	<---	antisense riboprobe
message	--->	synthetic message
d) primers	■	24-mer oligodeoxynucleotides
e) cDNA	▬	reverse transcribed from synthetic message
	- - — ▬	reverse transcribed from mRNA
f) amplified/ protected fragment	▨	240 n, β-actin 167 n, Hox 2.1

at 37°C for 30 min for the experiments shown in Figures 1, 4 and 5. For the experiment shown in Figure 3, non-hybridized riboprobe was digested by 2.5 μg/ml RNase A and 0.3 μg/ml RNase T_1 in 10 mM Tris-HCl (pH 8.0), 5 mM EDTA and 0.9 M NaCl at 37°C for 30 min. Protected fragments were separated by electrophoresis through denaturing polyacrylamide gels and were visualized by autoradiography.

RESULTS

During the course of our experiments on gene expression in preimplantation mouse embryos it became apparent that it is important to measure the number of copies of specific mRNAs in small numbers of early embryos. The strategy that we used to do this was to synthesize mRNA *in vitro* from sequences cloned into riboprobe vectors and then to use PCR to amplify varying amounts of specific regions of this RNA (corresponding to different numbers of transcripts per embryo). These amplified DNA fragments were then detected and quantified using an RNase protection assay (after hybridization with saturating amounts of riboprobe) to construct a standard curve. It was possible to accurately determine the number of mRNA molecules per embryo by comparing the amount of amplified fragments from the identical region of the message in RNA from a known number of embryos to the standard curve. Furthermore, since the number of cells per embryo is known for preimplantation stages, the number of mRNAs per cell can be determined, if the transcripts are uniformly distributed.

In performing this analysis, it is important to minimize the loss of product during the steps in the assay. One way to do this is to reduce the number of times the reaction product is transferred from tube to tube. Thus, reverse transcription and sequence amplification by PCR were attempted in a single-step reaction. Preliminary tests were performed in which one-step and two-step amplification reactions were compared. In the two-step reaction, 1 μg of total RNA from mouse liver was reverse transcribed from an oligo (dT) primer and, after purification, one-tenth of the cDNA product was amplified by Taq DNA polymerase either in the presence or the absence of MMLV reverse transcriptase. In the one-step reactions, 1 μg of total mouse liver RNA was reverse transcribed from the 3′ β-actin PCR primer, in the presence of Taq DNA polymerase and the 5′ primer, and then immediately amplified in the same tube. Amplification products from each reaction were hybridized to radioactively labeled antisense β-actin riboprobes and an RNase protection analysis performed.

The results of these tests are shown in Figure 1. Lanes a and b contain 20% of the one-step reactions, in which RT-PCR was performed starting from 1 μg of total RNA, in either 1×reverse transcription buffer (lane a) or 1×PCR buffer (lane b). Lanes c and d contain 20% of the two-step reactions, in which PCR was performed starting from the equivalent of 100 ng total RNA, either in the presence (lane c) or absence (lane d) of reverse transcriptase. The protected fragments in all four lanes were of the expected size of 240 n. When the intensities of the bands were measured by densitometry, the protected fragments for the one-step reactions were found to be equivalent. In the two-step reactions the band in lane d was about twice as intense as that in lane c, suggesting the possibility that the presence of reverse transcriptase inhibits PCR amplification to some extent. In the experiments shown here the signals from the one-step reactions are about twice that from the two-step reactions, although the amount of RNA amplified is 10-fold greater. The efficiency of the one-step reaction is, therefore, lower by about 5-fold. However, because the amplified products are qualitatively the same and because the overall sensitivity of the procedure exceeds what is required, we selected the one-step protocol for the quantitative analysis. The one-step protocol has the advantages that sample assembly is easier and that there are fewer manipulations that might result in differential loss of material.

To verify the accuracy of the quantification procedure, we chose initially to construct a standard curve to determine the amount of β-actin mRNA in 32-cell blastocysts. Synthetic messenger RNA (Figure 2c) was transcribed *in vitro* from a part of the β-actin mRNA sequence (Figure 2a) that is cloned into a riboprobe vector (Figure 2b). Varying amounts of synthetic message, from 32 to 3.2×10^6 mRNAs per embryo, were amplified by RT-PCR in one-step reactions (Figure 2e and f). A parallel reaction, containing RNA from one mouse blastocyst, was amplified simultaneously. Labeled antisense riboprobe (Figure 2b) was hybridized to denatured amplification products and subjected to RNase protection analysis. The protected fragments (Figure 2f) were separated by electrophoresis through denaturing polyacrylamide gels and visualized by autoradiography. The data shown in Figure 3 are from one of these experiments. (In total, this analysis was performed four times, three times using RNA from one blastocyst and once using RNA from 0.1 blastocyst.) In panels A and B, lanes b through g contain amounts of synthetic message that correspond to 32 through 3.2×10^6 messages per one blastocyst stage embryo, increasing by factors of 10-fold. Lane h contains total RNA from a single blastocyst. The 24 hr exposure in panel A allows visualization of the amplification products equivalent to 32 to 3200 mRNAs per embryo, while the 1 hr exposure in panel B shows the products from 3.2×10^4 to 3.2×10^6 mRNAs per embryo. The regions of the dried gel containing the protected fragments, as well as the corresponding region in the control lane (background), were excised into vials containing scintillation fluor and the radioactivity present in each was counted. When the data were expressed as the percentage of maximum counts and plotted against copy number per embryo, it was evident that the amplification of synthetic β-actin message was linear from 32 to 3.2×10^5 molecules per embryo. By then superimposing the signal from the β-actin mRNA from one embryo on this standard curve, it was determined that there are 2.5×10^5 messages per blastocyst. When the data from all four β-actin mRNA experiments were analyzed together, the mean of the copy number was $1.4\times10^5 \pm 0.4$ (S.E.M.). This number is in excellent agreement with that reported by Taylor and Piko (9) of $1.3\times10^5 \pm 0.2$ for β-actin mRNA. It should be pointed out, however, that our analysis could be detecting γ-actin mRNA to some extent, because the primers lie within the highly conserved protein-coding portion of the mRNA. In 32-cell blastocysts, 2/3 of the actin mRNA encodes β-actin and 1/3 γ-actin (9). Therefore, if we are detecting all of the γ-actin mRNA, our β-actin mRNA copy number would be lower than that of Taylor and Piko (9) by only about 30%, still agreeing very well with their result.

It should be noted that the β-actin mRNA reconstruction became non-linear between 3.2×10^5 and 3.2×10^6 mRNAs per embryo (Figure 3B). By performing these analyses with differing amounts of riboprobe, we determined that this non-linearity resulted from limiting amounts of riboprobe, which precludes saturation of all amplified DNA fragments (data not shown). It is, therefore, always necessary to use an amount of riboprobe that will hybridize to all amplified DNA fragments in the range of interest for the mRNA being tested.

In our studies aimed at determining whether two homeobox-containing genes (Hox 2.1 and Hox 3.1) are expressed in preimplantation stage embryos, we detected low levels of transcripts from Hox 2.1 in blastocysts. To determine the amount of this rare RNA in blastocysts, we applied the single-step

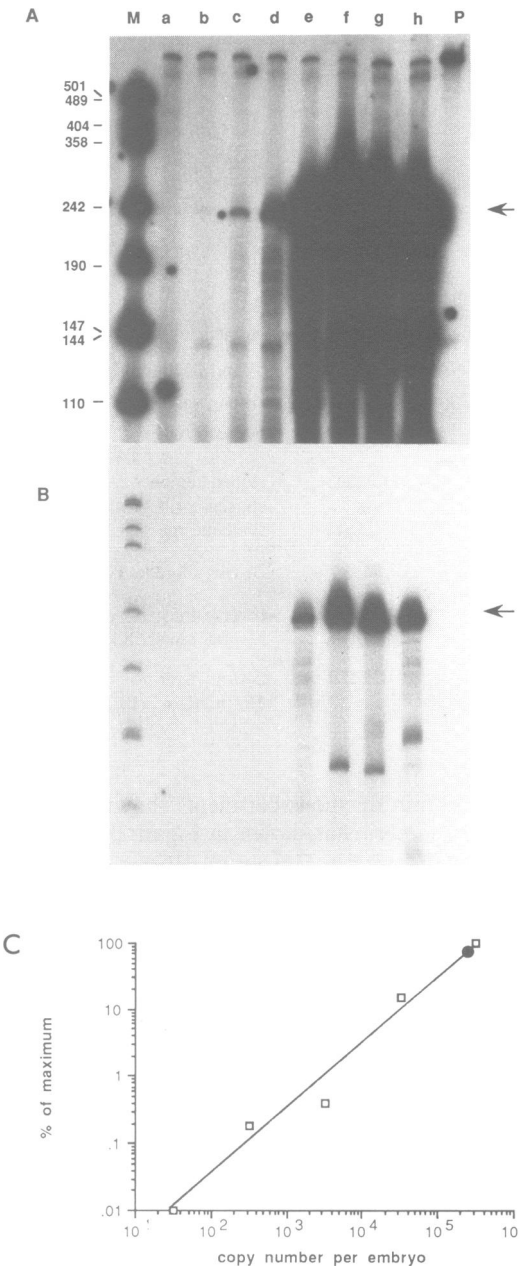

Figure 3. Quantification of β-actin mRNA in a single mouse blastocyst. Panels A and B show the detection of synthetic and natural β-actin mRNAs by RNase protection of β-actin riboprobe by PCR amplified DNA fragments. The lanes are as follows: a) digested probe, no added DNA; b) 32 copies of synthetic message per embryo (1 copy per cell); c) 320 copies per embryo; d) 3,200 copies per embryo; e) 32,000 copies per embryo; f) 320,000 copies per embryo; g) 3,200,000 copies per embryo; h) total RNA from one 3.5 day mouse blastocyst (32 cells); M) markers; P) undigested probe. Arrows indicate the protected fragments of 240 n. In this experiment, 5×10^5 dpm of riboprobe at a specific activity of 5.2×10^7 dpm/μg were hybridized to the amplified DNA fragments. Exposure times were 24 hr at −80°C using an intensifying screen (Panel A), and 1 hr at 22°C (Panel B). Panel C shows the standard curve that was constructed after counting regions of the dried gel containing the protected fragments. The corresponding region from the control lane was also counted and these counts were subtracted as background. Radioactivity was expressed as percent of maximum and was plotted against the calculated copy number of synthetic messages per embryo. □ synthetic message, ● endogenous β-actin message in a single blastocyst. (The position of this point on the curve was determined by correcting the number of counts in the band to allow for recovery of only 64% of the RNA from blastocysts.)

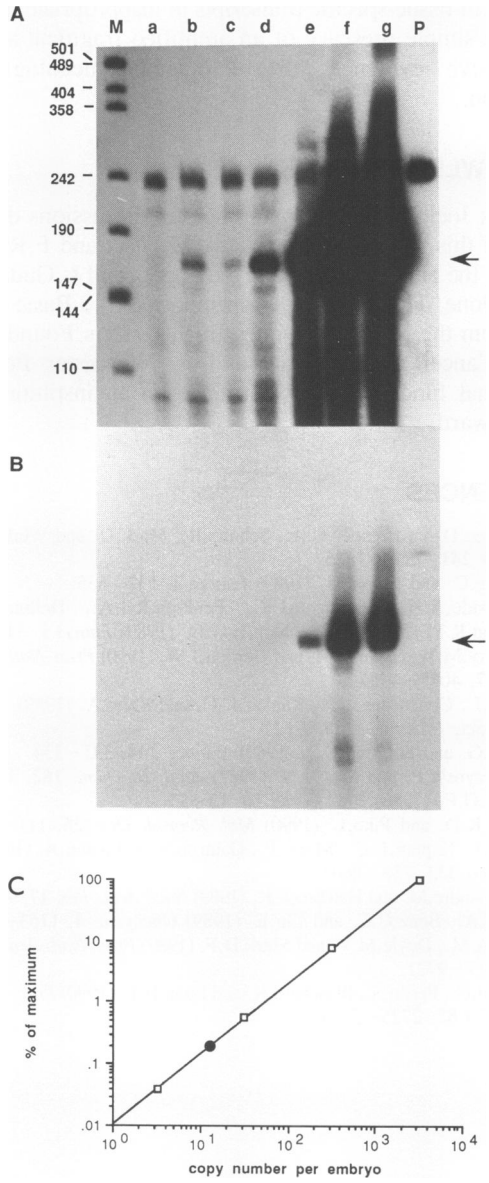

Figure 4. Quantification of Hox 2.1 mRNA in blastocysts. Panels A and B show the detection of synthetic and natural Hox 2.1 mRNAs by RNase protection of Hox 2.1 riboprobe by PCR amplified DNA fragments. The lanes contain: M) markers; a) digested probe, no added DNA; b) total RNA from ten early mouse blastocysts; c) 3.2 copies of synthetic message per embryo (this number is calculated for direct comparison to the 10 embryo equivalents in the blastocyst lane, as are the numbers in lanes d–g); d) 32 copies per embryo; e) 320 copies per embryo; f) 3,200 copies per embryo; g) 32,000 copies per embryo; P) undigested probe. Arrows indicate the protected fragments of 167 n. In this experiment, 8.4×10^4 dpm of riboprobe at a specific activity of 5.2×10^7 dpm/μg were hybridized to the amplified DNA fragments. Films were exposed for 48 hr at −80°C using an intensifying screen (Panel A) and 2 hr at −80°C (Panel B). Panel C shows the standard curve constructed after regions of the gel containing the 167 n fragment were excised, counted and the data plotted as for Figure 3C. □ synthetic message, ● endogenous Hox 2.1 message in ten blastocysts. (As in Figure 3, the counts in this band were corrected for a recovery of 64% of blastocyst RNA before placing the point on the standard curve.)

reconstruction assay (Figure 4). In this figure, lanes c through g of panels A and B contain 3.2 through 3.2×10^4 messages per embryo (matched for 10 blastocyst stage embryos) and lane b contains total RNA from ten early blastocysts. The long exposure in panel A shows the amplification products from 3.2 and 32

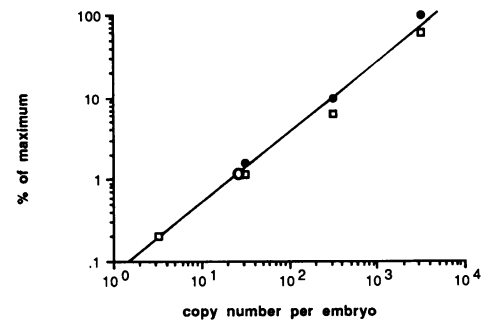

Figure 5. Reconstruction analysis of Hox 2.1 transcripts with and without the addition of blastocyst RNA to the synthetic message. A single-step reconstruction experiment was carried out as described in Figure 4, except that 5 rather than 10 embryo equivalents of RNA were used. The standard curve that was constructed after counting regions of the gel containing amplified fragments from synthetic Hox 2.1 message alone (□) and synthetic message plus total RNA from five blastocysts (●) is shown. The point with 3.2 copies of synthetic mRNA per embryo plus endogenous mRNA from five early blastocysts (○) was used to determine the number of Hox 2.1 transcripts (22) by subtracting 3.2 from the measured number of 25.

synthetic mRNAs per embryo and the shorter exposure in panel B, the products from 320 to 3.2×10^4 mRNAs per embryo. The regions of the gel containing the protected fragments (167 n) were excised and counted and the data were plotted (Figure 4C). In the example shown here, it was found that the amplification was linear from 3.2 to 3200 mRNAs per blastocyst and the Hox 2.1 message was present in early blastocysts at about 14 copies per embryo. This analysis was performed a total of three times yielding a mean copy number of 18 ± 3 (S.E.M.). As was seen in the actin mRNA experiments, the reconstruction analysis becomes non-linear at the highest amounts of synthetic RNA (between 3.2×10^3 and 3.2×10^4 mRNAs per embryo). This again was found to be due to limiting amounts of the riboprobe (data not shown). We were concerned that this low message number may have resulted from artifactual inhibition of reverse transcription or PCR amplification by some contaminant present in samples containing larger amounts of blastocyst RNA. To address this issue, two simultaneous Hox 2.1 reconstructions were performed. The first was a standard reconstruction set up to match the parallel amplification of total RNA from five early blastocysts. The second reconstruction contained total RNA from five early blastocysts in addition to the synthetic Hox 2.1 message in each reaction tube. The standard curves derived from this experiment are identical (Figure 5). When the signal from the 3.2 copies per embryo plus five blastocysts was superimposed on the standard curve, it was determined that there were 22 natural mRNAs per embryo in addition to the 3.2 synthetic messages per embryo. Whether this low amount of Hox 2.1 RNA in blastocysts is biologically important is unknown.

DISCUSSION

Several strategies for quantitating message levels using the polymerase chain reaction have been described previously (10, 11, 12, 13, 14). Some (10, 12) based quantitation upon coamplification of a reporter gene, which differs from the target gene. This may not be a reliable strategy since differences in primer efficiencies lead to differences in the final amount of product amplified. Another (14) compared the coamplification

of target cDNA to cloned genomic sequences. This strategy failed to account for the effects of reverse transcription as well as possible differences in the amplification efficiencies of genomic and complementary DNAs. Differences in size of the target sequences may also affect amplification, thus, the presence of introns in the genomic sequences may have affected quantification. In another strategy, an artificial internal standard was constructed that consisted of the 5′ and 3′ primer sequences of several target genes cloned into an Okayama–Berg vector that contained a T7 promoter and a polyadenylated sequence (13). Synthetic message was transcribed from the T7 promoter and was quantitated by spectroscopy. A known quantity of synthetic message cDNA was mixed with total RNA and serial dilutions of the mixture were reverse transcribed and coamplified. This method avoided the problem of different primer efficiencies. However, differences in reverse transcription can result from differences in nucleotide sequence, in the length of the poly (A) tail and in distance between the poly (A) tail and the PCR primers (13), all of which differed for the artificial standard relative to the endogenous sequence, and can lead to differences in the amount of product amplified. Strategies which controlled for these differences by coamplifying the target sequences with exogenous target sequences that have been altered by site-directed mutagenesis to insert or delete a restriction site (11, 14) were complicated by the possible formation of heterodimers between native and mutated amplified fragments during later amplification cycles. Since heterodimers are not recognized as substrates for the restriction enzymes, quantification was artificially skewed toward the uncleaved species.

In the experiments reported here, synthetic messages transcribed *in vitro* from cloned sequences were used, rather than the cloned DNA sequences themselves, thus controlling for reverse transcription and avoiding possible differential amplification of plasmid DNA and cDNA. Serial dilutions of these synthetic messages were reverse transcribed and amplified by PCR in parallel with reactions containing total RNA from early blastocysts. Since the standards were not coamplified with the experimental RNAs, there was no need to construct an artificial standard nor to mutagenize endogenous sequences. The effects of tube-to-tube variations were minimized by construction of the standard curve over several orders of magnitude and by mixing the components for amplification reactions in large batches and aliquoting constant volumes to the RNA and oligos in each reaction tube. In addition, the amplification reactions described here are carried out in single tubes with one reaction mix. There is no need to add PCR reagents subsequent to reverse transcription or to dilute the reaction before the final PCR cycle (11). Thus, this method is easy to use. In addition, the standard curve can be linear over a range of up to four orders of magnitude (Figure 3C), making this method equally valid for abundant as well as rare sequences. In the experiments reported here, we have detected as many as 3.2×10^5 mRNAs per embryo from a single embryo and as few as 3.2 mRNAs per embryo from ten embryos in the linear regions of the standard curves. The accuracy of this method is further verified by the good agreement between our result for β-actin mRNA and that of Taylor and Piko (9).

In summary, the method described in this paper is easy to use and interpret. It has been used to accurately measure the level of the previously quantitated β-actin mRNA and to measure the level of a rare transcript (Hox 2.1) in preimplantation embryos. Quantification will be a useful technique for the analysis of gene expression in preimplantation mammalian embryos since the detection of tissue-specific transcripts in inappropriate tissues has made the simple presence of an amplified fragment after PCR inconclusive evidence for biologically meaningful gene expression.

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