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Visualization of the Expression of HMGN Nucleosomal Binding Proteins in the Developing Mouse Embryo and in Adult Mouse Tissues

Takashi Furusawa and Michael Bustin

Abstract

Visualization of the expression pattern of specific proteins during development and in adult tissues provides important clues as to their possible role in various cellular processes. Mouse is the organism of choice for obtaining information on gene expression patterns in higher eukaryotes. This chapter describes the protocols we utilized to visualize *Hmgn* transcripts and HMGN proteins in mouse tissues. HMGN are chromatin-binding proteins that affect chromatin structure and function and play a role in cellular differentiation.

Keywords

Chromosomal proteins; HMGN protein; mouse embryogenesis; development; in situ hybridization; immunofluorescence

1. Introduction

Tissue differentiation during embryogenesis is an orderly, preprogrammed process involving multiple changes in gene expression and chromatin remodeling at specific loci. The dynamic remodeling of chromatin structure is facilitated by numerous chromatin-binding proteins which can impact development either globally or along a specific pathway. Information of the expression pattern of specific proteins during development and in adult tissues provides important clues as to their possible role in various cellular processes. Here we describe the methods we use to visualize the expression of members of the HMGN protein family in mice, a model organism widely used for studies on higher eukaryotes (1, 2). HMGN are a family of nucleosome binding proteins known to affect the structure and function of chromatin (3, 4).

This chapter describes the protocols we utilized to visualize HMGN protein family in the developing embryo and in adult tissues. We use whole mount in situ hybridization and in situ analyses of tissue sections to visualize HMGN transcripts and immunofluorescence to visualize HMGN proteins in tissue slices. Techniques for analyses of HMGN transcripts and proteins in tissue culture cells can be found elsewhere (2, 5). The methods are slight modifications of procedures described in detail in Wilkinson (1992) (6).

2. Materials

All materials for in situ hybridization should be RNase free made with DEPC-treated water. The washing buffers and solutions for post-hybridization do not need to be RNase free.

2.1. Whole Mount in Situ Hybridization

2.1.1. Embryo Fixation

1. 4% PFA/PBS: dissolve 20 g of paraformaldehyde (Sigma) in 500 mL of PBS at 65 °C. To the fully dissolved, clear solution, add 30 µL of 10 N NaOH. Keep 50 mL aliquots at −20 °C. Do not repeat freezing and thawing. Keep at 4 °C and use within 2 weeks.

2.1.2. Pre-hybridization and Hybridization

1. PBT: 0.1% Tween 20/PBS; add Tween 20 (Sigma) to PBS to a final concentration of 0.1%.
2. Proteinase K/PBT: add 10 mg/mL proteinase K (Roche) stock solution to PBT to a concentration of 10 µg/mL. Prepare freshly before use.
3. 4% PFA+0.2% glutaraldehyde/PBT: dissolve 4 g of PFA in 100 mL of PBS at 65 °C. After cooling to room temperature, add 0.8 mL of 25% glutaraldehyde (Sigma) and 0.1 mL of 10% Tween 20. Prepare freshly before use.
4. 20 × SSC pH 5: 175.3 g NaCl, 57.4 g 3 Na–Citrate.2 H₂O, 22.1 g citric acid. Add H₂O to 1 L. Autoclave.
5. Hybridization buffer: 50% formamide (Invitrogen), 5 × SSC pH 5, 1% SDS, 50 µg/mL yeast tRNA (Roche), 50 µg/mL heparin (Sigma). Prepare freshly before use.

2.1.3. Post-hybridization Wash and Antibody Reaction

1. Solution I: 50% formamide, 5 × SSC pH 5, 1% SDS. Prepare freshly before use.
2. Solution II: 10 mM Tris–HCl pH 7.5, 0.5 M NaCl, 0.1% Tween 20.
3. Solution III: 50% formamide, 2 × SSC pH 5. Prepare freshly before use.
4. RNaseA/solution II: prepare the stock solution of 10 mg/ mL RNaseA (Sigma R5500) according to manufacturer's instruction. Dilute 1:100.
5. TTBS: 1 × TBS + 0.1% Tween 20
6. 10 × TBS pH 7.5: 60.6 g Tris, 43.8 g NaCl, 33 mL concentrated HCl/500 mL. Autoclave.
7. 10% blocking reagent stock solution: dissolve 3 g of blocking reagent (Roche 1096176) in 30 mL of maleic acid buffer (100 mM maleic acid, 150 mM NaCl, adjust pH 7.5 with 10 N NaOH) at 65 °C. Keep 1.5 mL aliquots at −20 °C.
8. 1.5% blocking reagent: dissolve 1.5 mL of 10% blocking reagent stock solution in 8.5 mL of TTBS. Prepare freshly before use.
9. Embryo powder:
 - a. Collect embryos from pregnant mice (see **Note 1**).
 - b. Place the embryos into 50 mL conical tube. Add PBS 0.5 mL/embryo.
 - c. Homogenize embryos.
 - d. Add four volumes of ice cold acetone, mix with vortex, and incubate on ice for 30 min (mix occasionally during incubation).
 - e. Centrifuge at 4000 rpm for 10 min. Discard supernatant.
 - f. Spread pellet out on paper towel, let it dry thoroughly.

- g. Grind into a fine powder.
- h. Transfer the powder to 2.0 mL centrifuge tube. Store at -20°C .

2.1.4. Alkaline Phosphatase Reaction and Wash

1. NTMT: 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM MgCl_2 , 0.1% Tween 20.
2. Alkaline phosphatase (AP) buffer: dissolve 1 g of polyvinyl alcohol (Sigma 36.313-8 MW31000-50000) into mixture of 7.25 mL of water, 0.25 mL of 1 M Tris-HCl (pH 9.5), 0.2 mL of 5 M NaCl at 90°C for 20 min. Cool down slowly, add 0.5 mL of 1 M MgCl_2 , 0.1 mL of 10% Tween 20, 45 μL of NBT (4-nitro blue tetrazolium chloride; Roche), 35 μL of BCIP (5-bromo-4-chloro-3-indolylphosphate; Roche), and add water to 10 mL. Prepare freshly before use.
3. PBTE: PBT + 1 mM EDTA 2.2. In Situ Hybridization of Tissue Sections

2.2.1. Pre-hybridization and Hybridization

1. Acetylation buffer: 1 mL of concentrated HCl, 6 mL of triethanolamine (Sigma T1377), 400 mL of DEPC-treated water.
2. Hybridization buffer: same as whole mount in situ hybridization.

2.2.2. Post-hybridization Wash and Antibody Reaction

1. Solution I: 50% formamide, $5 \times \text{SSC}$ pH 5. Prepare freshly before use.
2. Solution II: 10 mM Tris-HCl pH 7.5, 0.5 M NaCl.
3. Solution III: 50% formamide, $1 \times \text{SSC}$ pH 5. Prepare freshly before use.
4. Buffer 1: 100 mM Tris-HCl pH 7.5, 150 mM NaCl

2.2.3. Alkaline Phosphatase Reaction and Wash

1. NTM: 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM MgCl_2 .
2. Alkaline phosphatase (AP) buffer: add 4.5 μL of NBT and 3.5 μL of BCIP to 1 mL of NTM. Prepare freshly before use.
3. PBSE: PBS + 1 mM EDTA

2.3. Immunofluorescence

2.3.1. Embryo Fixation

1. 10% formalin solution, neutral buffered (Sigma HT50-1-1)

2.3.2. Antibody Retrieve

1. Citrate buffer (pH 6.0): 8.2 mM $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, 1.8 mM $\text{C}_6\text{H}_8\text{O}_7$; to prepare 100 mL of citrate buffer, add 8.2 mL of 100 mM $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ stock solution and 1.8 mL of 100 mM $\text{C}_6\text{H}_8\text{O}_7$ stock solution to 90 mL of water.

3. Methods

3.1. Whole mount in situ Hybridization

3.1.1. DIG-Labeled RNA Probe Synthesis

1. Linearize 10 µg of template plasmid DNA (*see Note 2*) by digestion with 20 U of restriction enzyme in 100 µL reaction mix, at 37 °C for 2 h.
2. Check that the plasmid DNA is completely digested by electrophoresis. Take 1 µL and add 10 µL of 1 × loading buffer. Electrophoresis in 0.7% agarose gel.
3. Purify the DNA by phenol/chloroform/isoamyl alcohol (25:24:1) and precipitate by ethanol. Resuspend DNA in the 10 µL of DEPC-treated water (approximately 1 µg/µL). This linearized DNA can be stocked as a template for RNA probe at −20 °C.
4. Synthesize RNA probe at 37 °C for 2 h. For 20 µL reaction mix: 1 µg of linearized plasmid DNA, 1 × DIG RNA-labeling mix (Roche 1277073), 20 U T3, T7 or SP6 RNA polymerase (Stratagene 600111[T3], 600123[T7], 600151[SP6]), 1 × RNA polymerase buffer (Stratagene, comes with polymerase), 20 U RNase inhibitor (Invitrogen 10777019), 10 mM DTT.
5. Check the amount of synthesized RNA probe by electrophoresis. Take 1 µL and add 10 µL of 1 × loading buffer. Electrophoresis in 1.5% agarose gel (10–20 times more RNA is synthesized from DNA template)
6. During step 5, digest template DNA with DNaseI (Roche 104159). Add 20 U of DNaseI, 20 U of RNase inhibitor, and 20 mg of yeast tRNA and incubate at 37 °C for 10 min.
7. Add DEPC-treated water to 100 µL. Add 10 µL of 4 M LiCl₂ and 300 µL of ethanol. (Store at −20 °C before use).
8. Collect RNA by centrifugation. Wash pellet with 70% ethanol and briefly dry by micro-vac. Resuspend RNA in 50–100 µL of DEPC-treated water. RNA probe concentration is 0.1 µg/µL approximately. If probe length is more than 1 kb, alkali digestion is used to reduce the probe length (go to step 9 and also *see Note 2*).
9. Alkali digestion: precipitate probe and resuspend pellet in 200 µL of alkali buffer (40 mM NaHCO₃, 60 mM Na₂CO₃, pH 10.2) and digest at 60 °C for required reaction time. Formula of reaction time is as following: $T = (Li - Lf) / (k \times Li \times Lf)$, T is reaction time (minutes), Li is original length of probe (kb), Lf is desired length of probe* (kb), k is constant, 0.11 (cuts/ kb/min) (*for whole mount in situ hybridization, 0.5 kb).
10. Add 1 µL of acetic acid and mix well.
11. Add 20 µL of 3 M NaOAc and 560 µL of EtOH, mix well.
12. Keep the mixture at −80 °C for 1 h.
13. Precipitate and resuspend as in step 8.

3.1.2. Preparation of Mouse Embryo

1. Take out the embryos from pregnant mouse (*see Note 3*).
2. Wash embryo in the cold PBS briefly and put it in 4% PFA/PBS as soon as possible. Punctuate embryos by needle (*see Note 4*). Fix the embryo at 4 °C (*see Note 5*).

3. Wash embryo in the PBT at room temperature for 10 min. Dehydrate embryo (*see Note 6*) by sequential methanol/PBT treatment at room temperature for 10–60 min in each step: PBT, 25% methanol/PBT, 50% methanol/PBT, 75% methanol/PBT, and finally in 100% methanol. Repeat 100% methanol wash. Punctuate embryo again. Embryos can be kept for a few months in 100% methanol at -20°C .

3.1.3. Pre-hybridization Treatment and Hybridization

1. Rehydrate embryo by sequential methanol/PBT treatment at room temperature for 10–60 min in each step: 75% methanol/PBT, 50% methanol/PBT, 25% methanol/PBT, PBT (wash two times).
2. Decolorize embryo with 6% H_2O_2 /PBT at room temperature for 1 h.
3. Wash with PBT at room temperature for 10 min, two times.
4. Treat the embryo with 2–20 $\mu\text{g/mL}$ proteinase K/PBT (*see Note 7*).
5. Wash with PBT briefly and go to next step immediately.
6. Re-fix embryo by 4% PFA+0.2% glutaraldehyde/PBT at room temperature for 20 min.
7. Wash with PBT at room temperature for 10 min, two times.
8. Transfer the embryo to 2 mL tube (*see Note 8*). Add 1 mL of hybridization buffer. Pre-hybridization at 70°C for 1 h.
9. During step 8, precipitate and dissolve the RNA probe. Add probe to hybridization buffer to a final concentration of 1 $\mu\text{g/mL}$. Add 1.5 μg (15 μl) of probe to 0.5 mL of hybridization buffer.
10. Remove hybridization solution and add 1 mL of hybridization buffer. Add step 9 mixture. Hybridization at 70°C overnight.

3.1.4. Post-hybridization Wash and Anti-DIG Antibody Reaction

1. Wash with Solution I at 70°C for 30 min, two times. (Steps from here on do not need to be RNase free.)
2. Wash with Solution I: II (1:1 mixture) at 70°C for 30 min.
3. Wash with Solution II at 37°C for 5 min, three times (*see Note 9*).
4. Wash with Solution III at 37°C for 5 min.
5. Wash with Solution III at 65°C for 30 min, two times.
6. Wash with TTBS (+ 2 mM levamisole [Sigma L9756]) at room temperature for 5 min, three times.
7. Blocking: 1.5% blocking reagent/TTBS (+ 2 mM levamisole) at room temperature for 1–1.5 h. During this step, preincubate the antibody with embryo powder (Step 3.1.5). (*see Note 10*)
8. Remove blocking reagent. Add 1.25 mL of antibody solution at 4°C , o/n.

3.1.5. Pre-incubation of the Antibody (for 2 Samples)

1. Incubate 3 mg of embryo powder in 435 μL of buffer 1 at 70°C for 30 min.
2. Place the tube on ice.

3. Add 75 μ L of 10% blocking reagent, 1 μ L of anti-DIG-AP Fab fragment.
4. Incubate with rotation at 4 °C for 1 h.
5. Centrifuge at 3000 rpm for 2 min.
6. Take supernatant and mix with 2 mL of 1.5% blocking reagent/TTBS.

3.1.6. Alkaline Phosphatase Detection

1. Wash with TTBS (+ 2 mM levamisole) at 4 °C for 5 min, three times.
2. Wash with TTBS (+ 2 mM levamisole) at 4 °C for 1 h, five times.
3. Wash with TTBS (+ 2 mM levamisole) at 4 °C overnight.
4. Wash with NTMT (+ 2 mM levamisole) at room temperature for 10 min, three times.
5. Colorization with NBT-BCIP/AP buffer at room temperature. (Check the staining level every 10-20 min.)
6. To stop staining, wash with PBTE at room temperature for 10 min, three times. (Take the picture of the embryo. Further steps are optional.)
7. Re-fix the embryo with 4% PFA/PBS at room temperature for 30 min.
8. Wash with PBTE at room temperature for 10 min. Take the picture of the embryo (*see Note 11*).

3.2. In Situ Hybridization of Tissue Sections

3.2.1. Preparation of Paraffin Samples of Embryo

1. Prepare mouse embryo as in 3.1.1.
2. Place dehydrated embryo into xylene at room temperature for 1 h, two times.
3. Place embryo into melted paraffin (e.g., Paraplast Plus Tissue Embedding Medium, Fisher 23-021-400) at 55–60 °C for 1 h, two times. And then, place embryo into another paraffin at 55–60 °C overnight.
4. Embed the embryo into paraffin (*see Note 12*).
5. Cut paraffin block 0.4–0.6 μ m thick by microtome and put the sections on coated slide glass (KD Medical SSU-0050).
6. Dry the slide samples at 37–42 °C overnight.

3.2.2. Dewax to Hybridization

1. Remove paraffin and then rehydrate the section by sequential washing with CitriSolv (Fisher Scientific 22-143975) or with xylene (three times), 100% ethanol (three times), 90% ethanol/PBS, 70% ethanol/PBS, 50% ethanol/PBS, 30% ethanol/PBS, and PBS (two times), at room temperature for 5 min in each step (*see Note 13*).
2. Treat specimen with 1 mg/mL proteinase K/PBS at room temperature for 5 min.
3. Wash slide with PBS briefly and immediately move to step 4.
4. Fix specimen with 4% PFA+0.2% glutaraldehyde/PBS at room temperature for 20 min.
5. Wash slide with PBS at room temperature for 5 min (two times).

6. Wash with DEPC-treated water at room temperature for 5 min.
7. Treat the slide with acetylation buffer and 1 mL of acetic anhydride (Sigma A6404) (*see Note 14*).
8. Wash slide with PBS at room temperature for 5 min (two times).
9. Put 200 μ L of hybridization buffer on the slide and put the cover glass. Place the slide into the moisture box. Incubate at 70 °C for 1 h.
10. During step 9, precipitate and dissolve the RNA probe. Add probe to hybridization buffer to a final concentration of 1 μ g/mL.
11. Remove the cover glass and buffer. Put 200 μ L of hybridization buffer (from step 10) on the slide and put the cover glass. Place the slide into the moisture box. Incubate at 70 °C overnight.

3.2.3. Post-hybridization Wash and Anti-DIG Antibody Reaction

1. Wash with Solution I at 70 °C for 30 min, two times. (Steps from here on do not need to be RNase free.)
2. Wash with Solution II at 37 °C for 5 min, two times.
3. Digest excess amount of probe with 20 mg/mL RNaseA/ Solution II at 37 °C for 5 min.
4. Wash with Solution II at 37 °C for 5 min.
5. Wash with Solution III at 65 °C for 30 min, three times.
6. Wash with TTBS (+ 2 mM levamisole [Sigma L9756]) at room temperature for 5 min, three times.
7. Wash the slide with buffer 1 at room temperature for 5 min, three times.
8. Blocking with 1.5% blocking reagent/buffer 1 at room temperature for 1 h. During this step, pre-incubate the antibody with embryo powder (Step 3.2.4). (*see Note 15*)
9. Remove blocking reagent. Put 200 μ L of antibody solution on the slide and put the cover glass. Place the slide into the moisture box. Incubate at 4 °C overnight.

3.2.4. Pre-incubation of the Antibody (for 20 Samples)

1. Incubate 6 mg of embryo powder in 850 μ L of buffer 1 at 70 °C for 30 min.
2. Place the tube on ice.
3. Add 150 μ L of 10% blocking reagent, 2.5 μ L of anti-DIG-AP Fab fragment.
4. Incubate with rotation at 4 °C for 1 h.
5. Centrifuge at 3000 rpm for 2 min.
6. Take supernatant and mix with 4 mL of 1.5% blocking reagent/buffer 1.

3.2.5. Alkaline Phosphatase Detection

1. Wash with buffer 1 at room temperature for 20 min, three times.
2. Wash with NTM (+ 2 mM levamisole) at room temperature for 5 min, two times.
3. Colorization with NBT–BCIP/AP buffer by putting 200 μ L of buffer on the slide. Place the slide in the moisture box and keep at room temperature with shutting the

light. It will take 2–3 days to obtain the signal. Check with microscope until enough signals are detected.

4. Wash the slide with PBSE at room temperature for 10 min, three times.
5. Wash the slide with water at room temperature for 10 min, two times.
6. Embed the specimen with crystal mount (Biomedica M02). After embedding medium is dried, take the picture.

3.3. Immunofluorescence

3.3.1. Preparation of Mouse Embryo

1. Take out the embryos from pregnant mouse.
2. Wash embryo in cold PBS briefly and put it in 10% formalin buffer as soon as possible (*see Note 16*). Puncture embryos by needle. Fix the embryo at 4 °C overnight.
3. Wash embryo in PBS at room temperature for 10 min. Dehydrate embryo by sequential ethanol/water treatment at room temperature for 10–60 min in each step (*see Note 6*). At 70% ethanol, 90% ethanol, and finally in the 100% ethanol. Repeat 100% ethanol wash. Puncture embryo again.

3.3.2. Preparation of Paraffin Samples of Embryo

1. Place dehydrated embryo from previous step into xylene at room temperature for 1 h, two times.
2. Place embryo into melted paraffin (ex. Paraplast Plus Tissue Embedding Medium, Fisher 23-021-400) at 55–60 °C for 1 h, two times. And then, place embryo into another paraffin at 55–60 °C overnight.
3. Embed the embryo into paraffin.
4. Cut paraffin block 0.4–0.6 µm thick by microtome and put the sections on coated slide glass (KD Medical SSU-0050).
5. Dry the slide samples at 37–42 °C overnight.

3.3.3. Immunofluorescence

1. Remove paraffin and then rehydrate the section by sequential washing with CitriSolv or with xylene (3 times), 100% ethanol, 90% ethanol, 70% ethanol, and water, at room temperature for 5 min in each step.
2. Put the slide into citrate buffer and boil (in microwave oven) for 10 min (*see Note 17*).
3. Do not take the slide out from the buffer, leave and wait until it cools down to around room temperature (for 30 min).
4. Wash slide with PBS.
5. Remove the buffer on the slide as much as possible (do not dry the specimen), and circle the specimen by water replant marker such as ImmEdge PEN (Vector Laboratories H-4000).
6. Blocking: put 1.5% normal goat serum (Vector Laboratories S-1000)/PBS on the specimen and leave at room temperature for 30–60 min in a moisture box (*see Note 18*).

7. Primary antibody reaction: remove blocking buffer and put primary antibody mixture. For HMGN1 staining, 1/200–1/500 diluted anti-mouse HMGN1 rabbit polyclonal antibody is diluted in 1.5% normal goat serum/PBS. Incubate at 4 °C overnight.
8. Remove primary antibody mixture and wash slide by PBS at room temperature for 5 min, three times.
9. Secondary antibody reaction: put secondary antibody mixture. For HMGN1 staining, 1/200–1/500 diluted Alexa Fluor 488 goat anti-Rabbit IgG (Molecular Probes, A11008) is diluted in 1.5% normal goat serum/PBS. Incubate at room temperature for 1–2 h.
10. Remove secondary antibody mixture and wash slide with PBS at room temperature for 5 min, three times.
11. Counter stain the nucleus with Hoechst 33258 (Molecular Probes H3569), 500 ng/mL PBS. Incubate at room temperature for 10 min.
12. Wash slide by PBS at room temperature for 5 min, three times.
13. Mount the slide with mounting medium (ex. GEL/ MOUNT; Biomedica, MO1) and cover glass. After mounting medium is dried, seal the edge of cover glass with nail polish (Electron Microscopy Sciences, 72180)

4. Notes

1. We prepare embryo powder from E12.5 embryos.
2. In situ hybridization detects gene expression by the hybridization between mRNA and labeled nucleotide probe. Here we described the method using the RNA probe labeled with digoxigenin (DIG). RNA probe is synthesized by in vitro transcription using T3, T7, or SP6 RNA polymerase from cDNA template, therefore cDNA for a gene of interest is cloned into vector containing at least one of these promoters. To construct the template plasmid DNA, promoter position, cDNA length, and restriction enzyme site should be considered. Sense and antisense probes are generated from opposite direction of cDNA. Antisense probe hybridize with mRNA while sense probe can be used as control. We subcloned cDNA for *Hmgn* gene into pBluescriptII SK (Stratagene), which contains T3 and T7 promoter in opposite direction and multiple cloning sites in the middle. These plasmid can be used for both antisense and sense probe synthesis. For cDNA length, although recommended probe length is around 500 base pairs (bp) for whole mount and 150 bp for section in situ hybridization, longer RNA probe can be used for hybridization or can be digested after synthesis (*see step 3.1.1*). Restriction enzyme that generate 5'-hangover (such as *EcoRI*, *HindIII*, or *BamHI*) should be selected to facilitate polymerase activity. To use as a template, plasmid DNA has to be purified by CsCl ultra-centrifugation or QIAfilter (QIAGEN).
3. Embryos we used in this method for staining are from embryonic day 7.5 to 15.5-day-old embryos (E7.5–E15.5). Note that whole mount in situ hybridization is informative for tissues close to the surface of the embryos after E12.5 (*see Fig. 5.1*). To prepare embryo, please refer to other books (7).
4. To facilitate the probe penetration, puncture the embryo by needle (Fine Science Tools, 10130-20) on the head, trunk, heart, and back.
5. Fixation time length differs from embryo. Embryos E7.5–E8.5 for 1 h, E9.5 for 2 h, and E10.5 and older for overnight.

6. Dehydration/rehydration time lengths are changed according to the age of the embryo. Embryos E7.5–E8.5 for 10 min, E9.5 for 15 min, E10.5–E12.5 for 30 min, and E13.5 and older for 1 h.
7. Concentration of proteinase K and reaction time should be adjusted to the embryonic stage and the type of organ. For example, to detect the signal on the skin surface, E9.5–E10.5, digest with 10 $\mu\text{g}/\mu\text{L}$ for 5 min at 37 °C (or 10 min at room temperature), E11.5–E12.5, digest with 10 $\mu\text{g}/\mu\text{L}$ for 10 min at 37 °C (or 15 min at room temperature), and E13.5–E15.5, digest with 10 $\mu\text{g}/\mu\text{L}$ for 15 min at 37 °C (or 20 min at room temperature). For the E7.5–E8.5 embryo, proteinase K digestion is not necessary. To detect the signals in inner organs, longer treatment is required, but the embryo will be disintegrated during hybridization.
8. Embryos from E7.5 to E12.5, 2.0 mL centrifuge tube can be used for pre-hybridization and hybridization. For the embryos older than E13.5, use 15 mL tube and 6–8 mL of buffer.
9. For the first time staining, go to Section 3.1.4 step 4 immediately. After alkali phosphatase detection, if the back ground signal is too high, try to remove excess amount of probe by RNaseA digestion. Add 100 $\mu\text{g}/\text{mL}$ RNaseA/Solution II at 37 °C for 30 min. After digestion, wash with Solution II at 37 °C for 5 min and wash by Solution III at 37 °C for 5 min. Then return to step 5. Note that this RNaseA treatment also significantly reduces signal.
10. This pre-incubation step can be omitted. In that case, prepare antibody solution: dilute $\times 1/2500$ anti-DIG-AP Fab fragment (Roche 1093274) to 1.5% blocking reagent/TTBS (+2 mM levamisole).
11. To see the signals from internal organs, embryos can be cleared by glycerol. Incubate the embryo in the 50% glycerol/PBTE at room temperature for 1 h and then 80% glycerol/PBTE at room temperature for 1 h.
12. We use stainless base molds (Sakura/Tissue-Tek 4164) and plastic biopsy cassettes (Sakura/Tissue-Tek 4174) for embedding.
13. For histology staining (in situ hybridization of tissue sections and immunohistochemistry), we use Slide Holder (Sakura/Tissue-Tek 4465) for staining rack, Staining Dish White (Sakura/Tissue-Tek 4457) for ethanol and buffers, and Staining Dish Green (Sakura/Tissue-Tek 4456) for xylene.
14. Specimen is treated by acetylation buffer and acetic anhydride to remove charges of proteins (see Fig. 5.2). (i) Prepare 400 mL of acetylation buffer in a beaker and put a stirring bar in it. (ii) Place a staining rack by hanging on a bar with thread. Adjust the height of the staining rack to avoid from touching the stirring bar and make sure that the surface of buffer covers the specimen on the slides. (iii) Place the beaker on stirrer and start stirring. (iv) Fill 1 mL pipette with acetic anhydride. Place tip of the pipette close to the bottom of the beaker. Add only a few drops of acetic anhydride at the bottom of the beaker let it dissolve completely and after the drops completely disappeared, repeat this step until 1 mL of acetic anhydride is dissolved.
15. This pre-incubation step can be omitted. In that case, prepare antibody solution as follows: dilute $\times 1/2000$ anti-DIG-AP Fab fragment to 1.5% blocking reagent/buffer 1
16. For immunostaining (immunohistochemistry or immunofluorescence), fixation by 4% PFA/PBS is also used. For in situ hybridization, 4% PFA is recommended.

17. For antigen retrieval, we use Sakura/Tissue-Tek Slide Holder and Staining Dish White (as in **Note 13**). Fill the staining dish with 300 mL of citrate buffer, place the slide folder, and put a cover on it. Boil in a microwave oven (700 W) for 10 min. If the buffer is evaporated and specimen is not covered with buffer, stop the microwave oven and resume boiling after adding buffer to the staining dish.
18. For blocking, use serum from the same species as the secondary antibody. In this case we use goat anti-Rabbit IgG for secondary antibody, therefore normal goat serum should be used for blocking.

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Fig 5.1.

Expression patterns of Hmgn1, Sox9, and HMGN1 protein in the developing mouse embryo. (A–E) Whole mount in situ hybridization using mouse Hmgn1 antisense probe. Pictures of A–C are taken with microscopy and D–E are taken with digital camera that makes color appearance different (both AP staining, same substrate NBT-BCIP is used). (A) At E7.5 embryo, Hmgn1 is detected in the embryonic region (arrowhead) while no signal is detected in the ectoplacental cone (arrow). (A–F) As the embryo develops, the signal is detected only in the surface because of difficulty of probe penetration. (A'–F') Control experiments using mouse Hmgn1 sense probe. (G–K) The comparison of expression patterns of Hmgn1 (left) and Sox9 (right) in the developing limb bud. Hmgn1 expression is detected in the distal mesenchyme (filled arrowhead in G; left) and interdigit mesenchyme (filled arrowheads in H–J; left), while Sox9 is detected in the compensating mesenchyme (open arrow head in G; right) and digit mesenchyme (open arrowheads H–J; right). (L–O) Expression patterns of Hmgn1 in the histological sections of E14.5 embryo detected by in situ hybridization. (L) Section through neural tube. Hmgn1 is strongly expressed in the ventricular zone (filled arrow head), surface ectoderm (blank arrow head), and weakly expressed in the mantle layer (arrow). (M) Section through lung. Hmgn1 is strongly expressed in the distal epithelium (arrow head), while no expression is detected in the bronchiole (arrow). (N) Section through kidney. Hmgn1 is expressed interstitial mesenchymal cells around the urinary tubules (arrow head) and glomerulus (arrow). Expression level is stronger in the peripheral than in the central region of the kidney. (O) Section through eye (1). Strong expression of Hmgn1 is observed in the retina (arrow head) and lens epithelium, but not in the lens fiber (open arrow head). (L'–O') Control experiments using mouse Hmgn1 sense probe. No significant signals are detected. (P–R) The localization of HMGN1 protein is detected by immunofluorescence. Cell nuclei are counter stained with DAPI. (P, a and b) Sections through the E14.5 neural tube. HMGN1 is localized to the roof plate (rp), ependymal layer (el) of the neural tube, dorsal region of mantle layer (mL and (a) in P) but downregulated in the dorsal region of

mantle layer (b in P) and absent from marginal layer. mg, marginal layer; dg, dorsal root ganglia. (Q, c, and d) Sections through the developing mouse stomach at E16.5. HMGN1 expression is localized to basal layer of gastric mucosa (bl in (c)) and longitudinal muscle layer (lm in (d)). ls, lumen of stomach; pc, peritoneal cavity; lv, liver; cm, circular muscle layer. (R, e, and f) Sections through the lung. At E16.5, bronchial expression of HMGN1 was downregulated in the proximal region (br in (f)) and decreased in the mesenchymal region but remained strong in the distal region (de, distal epithelium in (e)); me, mesenchyme.

Fig. 5.2. Acetylation buffer treatment. This figure illustrates how to add 1 mL of acetic anhydride little by little. For detail, *see* **Note 14**.