A Colorimetric PCR Method for the Detection of 
*M. leprae* in Skin Biopsies from Leprosy Patients

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*Mycobacterium leprae*, the etiologic agent of leprosy, remains one of the few pathogens that cannot be cultivated *in vitro*. The diagnosis of leprosy is, therefore, still based upon principles used a century ago: clinical examination of the patient's lesions, demonstration of acid-fast bacilli (AFB) in slit-skin smears, and histopathology. Although in the majority of cases the diagnosis is clear cut, in early disease or in the indeterminate forms of leprosy clinical evidence may be inconclusive and AFB difficult to find. Furthermore, AFB can be observed only if they are present in a concentration of approximately \(10^4 \text{/mL}\). For the diagnosis of early disease, initiation of effective treatment and monitoring response to chemotherapy, there is a need to develop a method that could specifically detect small numbers of AFB \(<10^4 \text{/mL}\).

Owing to its remarkable sensitivity, the polymerase chain reaction (PCR) has an immense potential in the diagnosis of infectious diseases, particularly where the causative agents are difficult to culture. PCR tests for the diagnosis of leprosy have been developed where single pairs of primers amplify sequences within the genes encoding 18-kDa (\(^1\)), 36-kDa (pra) (\(^8\)), 65-kDa (\(^7, 20\)) antigens and rRNA (\(^1\)). In addition Woods and Cole (\(^9\)) have developed a test based on a repetitive sequence (RLEP) which is repeated 28 times in the *M. leprae* chromosome. These tests can detect between 10 to 100 bacilli and are far more sensitive than any other available method.

The sensitivity and specificity of PCR can be improved further by an additional reaction which follows the first round of amplification. This approach utilizes primers which hybridize to sequences internal to the original primers (\(^12\)). The nested strategy has an advantage because of the addition of the fresh reagents, the greater number of cycles, and dilution of amplified products reduce the inhibitory factors present. However, it involves extra manipulations, is more expensive, and more prone to contamination. One way of getting around some of these problems is by using a one-tube nested (OTN) PCR (\(^10\)) which circumvents the requirement for opening the tubes to add the fresh reagents and thereby reduces the risks of contamination; moreover there is only one reaction so it involves fewer manipulations and is more cost-effective.

The use of PCR in a routine diagnostic setting depends upon its speed, sensitivity, reliability and ability to screen large numbers of samples. However, the current method of detection of PCR products is unsuitable for large-scale screening. Different methods have been developed which have simplified the detection and have thus enhanced the utility of PCR in routine diagnostic applications. These methods rely upon the incorporation of modified primers into PCR products to readily allow immobilization and subsequent detection of the amplified products by the development of color. One such colorimetric method has already been developed in conjunction with OTN PCR for the detection of *M. tuberculosis* (\(^19\)).

In this report we describe the development of OTN PCR based on the detection
of a target within the RLEP sequence of *M. leprae*. The repetitive nature of this target gives it an advantage in terms of sensitivity over systems detecting single-copy targets. Furthermore, the method of detection of amplified products was simplified to enable large-scale screening of the samples. The usefulness of OTN PCR in the detection of small numbers of *M. leprae* in tissue samples was determined also. In addition, the method was made quantitative by the use of limiting dilutions and applied to monitoring of the efficacy of multidrug therapy (MDT) in leprosy.

**MATERIALS AND METHODS**

**Patients' samples.** Punch biopsies (4 mm) were collected from untreated leprosy patients presenting at the Marie Adelaide Leprosy Centre, Karachi. The biopsies were snap-frozen in liquid nitrogen and stored frozen until use. Patients were classified according to the Ridley and Jopling scale (13). Punch biopsies were also obtained from 7 LL/BL patients and 1 indeterminate patient receiving MDT, prior to treatment and at 3-, 6-, 12-, and 24-month intervals after the start of therapy. The nonendemic samples consisted of frozen skin biopsies from 8 patients with a variety of other nonleprosy disorders receiving treatment at University College Hospital, London.

**M. leprae DNA preparation for PCR.** Genomic *M. leprae* DNA was kindly provided by Dr. M. J. Colston (NIMR, London). The *M. leprae* DNA was diluted in sterile distilled water to achieve concentrations from 1 ng to 100 ag/2.5 pl. Cryostat AS 600 (Anglia Scientific, Cambridge, U.K.) was used to cut 5-μm sections of each biopsy. The cryostat was cleaned thoroughly between each sample. Individual frozen sections were resuspended in PCR buffer containing 0.5% Tween-20 and 100 μg/ml proteinase-K (Boehringer Mannheim, Lewes, U.K.) and incubated at 60°C for 1 hr. This was followed by 10 consecutive freeze boil cycles (20). Fifty μl of chloroform were emulsified with each sample and then the tubes were centrifuged for 2 min at 13,000 rpm. A 5-μl aliquot from the aqueous layer was used in a 25-μl amplification reaction.

**PCR primers.** Primers PS3 and PS4 hybridize to a specific sequence within the RLEP sequence of *M. leprae* and generate a 455-bp fragment. Primers NO3 and NO4 bind specifically to sequences internal to the outer primers and generate a 320-bp fragment. Both of these primer pairs were designed using the PRIMER software (Version 0.5; Lincoln, S. E., Daly, M. J., and Lander, E. S.; Whitehead Institute for Biomedical Research, 1991) and synthesized using phosphoramidite chemistry on a Gene Assembler Plus (Pharmacia Biotech Ltd., Milton Keynes, U.K.). Sequences for primers are: PS3, 5'-GGA CAC GAT TAG CGC GGC GCA CGT; PS4, 5'-TTG TGG TGG TGG GCT GTG GGG GTG TGG; NO3, 5'-TGC TGA AGG CGA TAT; NO4, 5'-AGG TTG CCG TAT GTG. Primers NO3 and NO4 were labeled at their 5' end with digoxigenin (British Biotechnology Ltd., Abingdon, U.K.) and biotin, respectively.

**Hot start PCR.** Hot start was accomplished by overlaying a mixture of all four primers in a reaction tube with a wax pellet. The wax was melted by incubation at 80°C for 10 min. After a brief centrifugation, the wax was allowed to solidify at room temperature to form a physical barrier above the primers. The other reaction components were layered on top of the wax and only mixed with the primers during the first denaturation step when the wax melted and the two layers were able to mix.

**PCR.** A 5-μl aliquot of each sample was assayed in a 25-μl reaction containing 10 mM Tris-HCl pH 8.8; 50 mM KCl; 1.5 mM MgCl2; 0.1% Triton X-100; 200 μM each of dATP, dCTP, dGTP, and dUTP; 4% of dimethylsulfoxide; 0.75 nmol of each of the outer primers; 12.6 nmol of each of the inner primers; and 0.75 units of Taq polymerase (Bioline, London, U.K.). All of the reactions were carried out on a Hybaid TR2 thermal cycler with the temperature monitored using a thermocouple in a dummy tube.

**OTN PCR.** For OTN PCR the cycler was programmed to amplify DNA in two different stages. The first stage consisted of 30 cycles of annealing at 68°C for 30 sec, during which a 455-bp fragment was generated by the outer primers. During the second stage, 20 cycles of annealing at 50°C for 30 sec were performed, resulting in the amplifi-
cation of a 320-bp nested fragment by the two inner primers. An initial denaturation temperature of 94°C for 3 min and subsequent denaturation and extension temperatures of 93°C for 30 sec and 72°C for 90 sec, respectively, were used throughout.

Specificity of the primers. The specificity of both the primer pairs for *M. leprae* DNA was tested separately. DNA was extracted from 18 different mycobacterial species: *M. asiaticum*, *M. avium*, *M. bovis*, *M. chelonei*, *M. duvalli*, *M. flavescens*, *M. fortuitum* (*peregrinum*, *ranae*), *M. gordonae*, *M. marinum*, *M. nonchromogenicum*, *M. phlei*, *M. scrofulaceum*, *M. smegmatis*, *M. terrae*, *M. tuberculosis*, *M. ulcerans*, *M. vaccae*, and *M. xenopi*. The outer primer pair of PS3 and PS4 was tested at two different concentrations, 4 nmol and 0.75 nmol; the amplification program consisted of denaturation at 94°C for 3 min and 50 cycles of 30 sec at 93°C, 30 sec at 68°C, and 90 sec at 72°C, with a final incubation at 72°C for 10 min. In a separate experiment, primers were tested with the OTN PCR conditions described previously but without the inner primers. The specificity of the inner set of primers was similarly tested for 50 cycles at an annealing temperature of 50°C and in a mock OTN PCR without the outer primers.

Inhibition controls. The presence of inhibitors in the clinical samples was determined by a method described previously (19).

Quantitation of PCR by limiting dilution. Samples from patients being treated with MDT were first diluted 1/5 in sterile distilled water and then serial twofold dilutions of the samples were made. The highest dilution showing amplification was taken as the PCR titer.

Colorimetric detection of PCR product. The method for colorimetric detection described by Wilson, *et al.* (19) was adapted for the detection of *M. leprae*. Microtiter plate wells were coated with 10 µg/ml of avidin (Sigma Chemical Co., Poole, U.K.) in 50 mM carbonate buffer (pH 9.6) for 1 hr at 37°C. The reaction tubes were heated to 80°C to allow the wax to melt, 200 µl of Tris-buffered saline pH 7.5 with 0.5% Tween-20 (TBS-Tween 20) was added to each tube. After washing the microtiter plates twice with TBS-Tween 20, 100 µl of each diluted product was added to the individual wells in duplicate, and the plates were incubated for 30 min at room temperature. The plates were washed twice with TBS-Tween 20 and then 100 µl of 1/1000 of anti-digoxigenin-alkaline phosphatase conjugate (Boehringer-Mannheim) in TBS-Tween 20 was added. After a 30-min incubation at room temperature the plates were washed four times with TBS-Tween 20 and once with 50 mM carbonate buffer pH 9.6; 100 µl of 1 mg/ml para-nitrophenol substrate (Sigma) in 50 mM carbonate buffer pH 9.6 and 1 mM of MgCl2 was added, and the optical density (OD) was read after 1 hr at 405 nm.

RESULTS

OTN PCR. In order to obtain the greatest sensitivity, the most suitable target for PCR in *M. leprae* is the RLEP sequence, which is present in 28 copies in the genome (21) and which has previously been used by Woods and Cole (20). When we initially rechecked the specificity of the primers (C and D) described in that study, we found cross-reactivity in the DNA from other mycobacterial species. In some cases bands of a size different from *M. leprae* were seen, but bands of a similar size were observed with DNA from *M. fortuitum-ranae* and *M. ulcerans*. This suggests both nonspecific amplification and the presence of elements related to the RLEP sequence in some species. We, therefore, designed new primers for an OTN PCR: 24-bp outer primers (PS3/PS4) and 15-bp inner primers (NO3/NO4). The positions of the different primer pairs are shown in Figure 1. PS3/PS4 amplified a 455-bp fragment of *M. leprae* DNA and 320-bp of this sequence was amplified by NO3/NO4.

The specificity of each primer pair alone was tested rigorously, using 1 ng of genomic DNA from different species and 50 cycles of PCR. Initial experiments showed some nonspecific amplification, but the use of a hot-start procedure (19) greatly improved the results (data not shown). All subsequent experiments therefore were carried out using hot-start. This also helped prevent the formation of primer dimers, which is crucial in the use of OTN PCR.

With the outer primer pair of PS3 and PS4, no amplification was observed with DNA from 18 mycobacterial species other than *M. leprae*. However when the anneal-
Fig. 1. Diagrammatic representation of the positions of different RLEP primer pairs. PS3/PS4 and NO3/NO4 are outer and inner primers used in this study and generate 455-bp and 320-bp fragments, respectively. Primers C and D were described elsewhere (20).

The sensitivity of the PCR was determined by the amplification of genomic M. leprae DNA dilutions ranging from 1 ng to 100 ag. One fg of DNA was routinely detected (1 genome corresponds to 5 fg), a sensitivity considerably greater than we had found using a single-copy sequence, where sensitivity was between 10 fg and 100 fg (9).

Colorimetric detection of PCR products. Agarose gel electrophoresis is not a convenient assay for screening large numbers of samples. We therefore adapted the OTN PCR for colorimetric detection. The internal primers NO3 and NO4 were labeled with digoxigenin (Dig) and biotin, respectively, at their 5' ends. OTN PCR therefore produced a 320-bp band which was labeled with a different ligand at each end. These molecules were captured in avidin-coated mi-

Fig. 2. Specificity of the primers; amplified products were analyzed on 1.8% agarose gel. a = Primers NO3/NO4 were used to amplify 1 ng of DNA from M. asiaticum (lane 2), M. avium (lane 3), M. xenopi (lane 4), M. fortuitum-peregrinum (lane 5), M. nonchromogenes (lane 6), M. tuberculosis (lane 7), M. bovis (lane 8), M. tuberculosi (lane 9), M. ulcerans (lane 10), M. scrofulaceum (lane 11), M. flavescens (lane 12), M. smegmatis (lane 13), M. leprae (lane 14). Lane 1 shows the 123-bp DNA ladder used as a size marker. b = A combination of PS3/PS4 and NO3/NO4 were used to amplify 1 ng of DNA from lanes 2–14 (same as 2a), M. phlei (lane 16), M. fortuitum-ranae (lane 17), M. gordonae (lane 18), M. fortuitum (lane 19), M. leprae (lane 20), M. duvalii (lane 21), M. terrae (lane 22), M. vaccae (lane 23), M. marinum (lane 24), M. tuberculosis (lane 25), M. leprae (lane 26), negative controls (lanes 27 and 28). Lanes 1 and 15 show 123-bp DNA ladder used as size marker.
Detection of *M. leprae* DNA in frozen skin biopsies. The OTN PCR was next used to test clinical samples: 5-μm sections from frozen biopsies taken from 40 untreated leprosy patients were processed and checked for the presence of inhibitors, and aliquots were then analyzed by OTN PCR. The diagnoses of the patients ranged across the whole leprosy spectrum. Four sections from each biopsy were tested, and amplified products were detected by agarose gel electrophoresis and color detection. Samples were counted as positive if *M. leprae* DNA was detected in any of the four sections. *

*M. leprae* DNA was detected in all the multibacillary sections (BI = 2 or above; N = 17). In paucibacillary patients, 69% of the samples (N = 13) with a BI of 1 to 1.5 and 70% of the samples (N = 10) with a BI of 0 were also positive (Fig. 3).

Not all sections from the same biopsy were positive. In the samples with the highest BIs (4–5.5), all sections were positive by PCR; in samples with BIs of 2–3.5, between two and four sections out of four were positive; and in samples with BIs of 0–1.5 only, one of the four sections was positive in most cases that showed amplification. All negative controls (N = 105, containing sterile distilled water instead of clinical sample) and eight skin-biopsy sections from patients with conditions other than leprosy remained negative.

**Effect of chemotherapy.** We have previously used limiting dilution PCR (LDPCR) to quantitate *M. leprae* DNA in sections of frozen skin biopsies. This study was used to determine the effect of MDT on *M. leprae* viability by following multibacillary patients for 2 years (9), hypothesizing that *M. leprae* DNA is broken down rapidly after the death of the organism. We found that in approximately half of the cases, *M. leprae* DNA became undetectable by PCR of the single copy *pra* gene (9). Since the RLEP PCR described here is more sensitive, we re-analyzed these samples (The Table). Using RLEP as a target, amplification was observed with some previously negative samples, although 7 out of 8 still became negative by 24 months. The one exception which stayed positive throughout had previously appeared anomalous, in that it was negative at 3, 6 and 12 months, but positive at 24; using RLEP, we detected *M. leprae* DNA at all time points. These results support our earlier findings, that the bacilli had all been killed by 24 months in half of the multibacillary patients, and that DNA is degraded so that even a multicopy repeated se-

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**FIG. 3.** Sensitivity of OTN PCR colorimetric method in detecting *M. leprae* from the skin biopsy sections of leprosy patients. Optical densities (ODs) of the samples from leprosy patients with varying bacterial indexes (0–5.5; N = 40) and nonendemic controls (NEC; N = 8) measured at 405 nm.
THE TABLE. Comparison of PCR titers determined by using pra gene and RLEP sequence as targets in samples from patients being treated with MDT.

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* Frozen skin sections collected from patients being treated with MDT at Marie Adelaide Leprosy Centre, Karachi.

b BI (bacterial index) = number of AFBs measured on a logarithmic scale.

c The greatest dilution at which amplification was observed using pra gene as a target; a titer of 1 indicates that amplification was observed only when the sample was run undiluted.

d The greatest dilution at which amplification was observed using RLEP sequence as a target; a titer of 1 indicates that amplification was observed only when the sample was run undiluted.

e Highest dilution at which PCR was still positive.

f Patient was histologically characterized as indeterminate.

sequence becomes undetectable, while in other patients a small number of bacilli persist.

DISCUSSION

Although established multibacillary leprosy can be diagnosed easily using clinical examination and microscopy, the diagnosis of early disease, indeterminate forms, and cases in whom the bacilli are difficult to demonstrate, continues to be a problem. There is, therefore, a need to develop a sensitive and specific method for rapid and definitive identification of M. leprae in cases whose clinical and histological findings are equivocal.

We have developed an OTN PCR for a sequence within the repetitive element of M. leprae. This sequence has been reported to be specific for M. leprae by hybridization (2). However, when using PCR, we found both nonspecific amplification and what appeared to be specific amplification, giving rise to bands of the expected sizes with some species. Since PCR can be effective with relatively small regions of homology compared to filter hybridization, these results are not incompatible. In order to verify the specificity, the two primer pairs were tested alone as well as in a combination. Some crossreactivity was observed with single sets of primers, but the nested reaction was specific. Clearly, if related sequences do exist in other species, their characterization and sequencing will allow more specific primers to be designed.

Since the repetitive element occurs in 28 copies in the genome, its use as a target has given the system an advantage over methods using single-copy targets in terms of sensitivity. Woods and Cole (20) have shown the RLEP sequence to be 15-fold more sensitive after PCR than a single copy se-
The sensitivity of our assay has been consistently 1 fg, as determined by a colorimetric method as well as agarose gel analyses of purified *M. leprae* DNA. Colorimetric methods for the detection of *M. leprae* have been described by another group (15, 16) with a detection limit of 100 fg to 125 fg of genomic DNA.

In previous studies using PCR based on *pra* (17) and RLEP (22) 56% and 73%, respectively, of samples with no detectable bacilli on microscopy were shown to be PCR-positive. Our results agreed with the previous reports, finding 70% of such samples to be positive, as well as 69% of samples with BIs of 1-1.5. How can the 30% of PCR-negative samples be explained? A variety of factors can influence the PCR result of sample with BIs of 1 or 0; the area chosen for biopsy may not be representative of the lesion or bacilli, if present, had completely degraded DNA which could not be amplified. Patients may not have had leprosy or AFB seen on microscopy may not have been *M. leprae*. AFB seen in lesions of leprosy patients (18), although there is no significant evidence for false-positive smear results in leprosy caused by cultivable mycobacteria. Alternatively, the DNA extraction and amplification methodologies may need to be improved further.

In most paucibacillary samples, not all of the sections gave positive PCR results. This would not be surprising if the number of bacilli is very low. Other groups have pooled multiple sections prior to DNA extraction (22), or used a single section, but amplified a larger proportion of the extracted DNA (17).

In order to determine the efficacy of MDT in leprosy, quantitative OTN PCR was applied to the samples collected from patients prior to and at regular intervals after the start of MDT. The titers obtained were compared to those we had observed with the *pra* gene as a target. Our results showed that previously negative samples were PCR-positive, and the titers were generally higher with primers targeting the RLEP sequence. This could be due to the higher detection limit of the assay which was consistently 1 fg compared to 10 fg to 100 fg observed with the *pra* gene as a target. Any sample with intact *M. leprae* DNA <100 fg but >1 fg would therefore be detectable by an assay using RLEP sequence and not *pra* gene as a target. The other possibility could be that if some portion of DNA from the dead or decomposed *M. leprae* can serve as a suitable template, the likelihood of amplification would be higher with primers targeting a multicopy sequence. Although a relationship between positive PCR results and potentially viable *M. leprae* has been suggested by several workers (5, 9, 20), well controlled studies are needed to determine the exact relationship between viability and positive PCR signals. Another important issue that requires investigation is the time interval that elapses between the biological death of *M. leprae* and either autolytic or host-induced degradative changes in the bacillary DNA which result in the loss of signals for PCR. Studies by Williams, et al. (18) have shown that whereas the mouse foot pad assay indicated that *M. leprae* lose viability after only 2 weeks of treatment, the changes that rendered the *M. leprae* DNA nonamplifiable required at least 2 months.

We and other investigators (6, 18) have demonstrated previously that PCR could be used for monitoring the efficacy of drug therapy in leprosy. In the present study we have again shown that potential by using a more-sensitive method. Relapse in the first documented case following MDT was caused by drug-sensitive persisters rather than the development of drug resistance (4). The persisters, because of their dormant state, are protected from the effect of drugs and resume multiplication as soon as the drugs are withdrawn. When MDT was introduced it was hypothesized that any post-MDT relapses would be due to the multiplication of drug-susceptible persisters. Currently, the only reliable method available for the detection of persisters is growth in thymectomized and irradiated mice (21), which is not only expensive but is extremely time consuming. The use of PCR would be a cheap and rapid alternative.

The application of this PCR protocol to a limited number of clinical samples shows the potential of this assay for large-scale screening. The OTN PCR described here is relatively inexpensive, costing half as much as standard nested PCR, and uses detection equipment required for enzyme-linked immunosorbent assays (ELISAs) which are
already available in many laboratories throughout the world, including those in developing countries.

SUMMARY
A one-tube nested polymerase chain reaction (PCR) method for the diagnosis of paucibacillary leprosy was developed using the repetitive RLEP sequence as a target. Detection of the PCR products was simplified by the adaptation of a colorimetric method. The test was specific for Mycobacterium leprae, and the sensitivity of the assay was 1 fg of purified genomic M. leprae DNA (less than one genome). Complete concordance was seen between the development of color and resolution on agarose gels. The results of frozen skin sections from untreated patients showed that the assay could detect 100% of multibacillary samples [bacterial index (BI) of 2 or more] and 69% and 70% of the samples with BIs of 1 and 0, respectively. The use of one-tube nested PCR in assessing the effectiveness of multidrug therapy (MDT) in leprosy also was determined. The simplified colorimetric assay was found to be sensitive, rapid and specific, and is suitable for use in routine diagnostic laboratories.

RESUMEN
Se usó la técnica de la reacción en cadena de la polimerasa (PCR) para establecer el diagnóstico de la lepra paucibacilar utilizando como blanco la secuencia repetitiva RLEP. La detección de los productos amplificados por PCR se simplificó utilizando un método colorimétrico. La prueba fue específica para Mycobacterium leprae, y la sensibilidad del ensayo fue de 1 fg de DNA genómico purificado de M. leprae (menos de un genoma). Se observó una completa concordancia entre el método colorimétrico y la resolución en geles de agarosa. Los resultados de la técnica PCR utilizando secciones congeladas de piel de pacientes con lepra no tratada, mostraron que el ensayo pudo detectar el 100% de las muestras multibacilares (índice bacteriano, IB, de 2 o más), y el 69% y 70% de las muestras con IBs de 1 y 0, respectivamente. También se determinó la utilidad de la técnica PCR para establecer la efectividad de la poliquimioterapia en la lepra. El ensayo colorimétrico simplificado fue sensible, rápido y específico; es, por lo tanto, adecuado para su uso rutinario en los laboratorios de diagnóstico.

RÉSUMÉ
Une méthode de réaction de polymérase en chaine (PCR) en un tube pour le diagnostic de la lèpre paucibacillaire a été développée en utilisant la séquence répétitive RLEP comme cible. La détection des produits de la PCR a été simplifiée par l'adaptation d'une méthode colorimétrique. Le test était spécifique pour Mycobacterium leprae, et la sensibilité du test était de 1 fg d'ADN génomique purifié de M. leprae (moins d'un génome). Une concordance complète a été observée entre le développement de couleur et la résolution en gels d'agarose. Les résultats des coupes cutanées réfrigérées provenant de malades non traités ont montré que le test pouvait détecter 100% des échantillons multibacillaires [indice bactérien (IB) de 2 ou plus] et 69% et 70% respectivement des échantillons avec un IB de 1 et 0. L'utilité de la PCR en un tube a également été déterminée pour évaluer l'efficacité de la polychimiothérapie (PCT) de la lepre. Le test colorimétrique simplifié a été trouvé sensible, rapide et spécifique, et convient à l'emploi dans des laboratoires de diagnostic de routine.

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